FINE MAPPING AND CHARACTERIZATION OF THE *iap* GENE IN SORGHUM [*Sorghum bicolor* (L.) Moench]

A Dissertation

by

JOHN ROBERT GILL

Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Chair of Committee,	William L. Rooney
Committee Members,	Patricia E. Klein
	David M. Stelly
	Jorge A. da Silva
Head of Department,	David D. Baltensperger

August 2014

Major Subject: Plant Breeding

ABSTRACT

The production of interspecific or intergeneric progeny using sorghum [*Sorghum bicolor* (L.) Moench] is greatly enhanced by the presence of the *iap* (*Inhibition of Alien Pollen*) allele. Hybridization between *S. bicolor* homozygous for *iap* and divergent species of sorghum and sugarcane (*Saccharum* spp.) has been demonstrated with introgression occurring in hybrids between *S. bicolor* and *S. macrospermum*. The intergeneric F_1 hybrids between sorghum and sugarcane were male and female sterile, so the plants were doubled with colchicine in an attempt to restore fertility. The objectives of this research were to determine the viability of the sorghum × sugarcane amphidiploids as a bioenergy crop, determine the optimum humidity for maize (*Zea mays* L.) pollen tube growth on the pistils of sorghum homozygous for *iap*, and fine map the *Iap* locus and identify candidate genes. Sorghum × sugarcane amphidiploids were evaluated in a yield trial in Weslaco, TX. Although the amphidiploid genotypes were inferior to the intergeneric F_1's for most agronomic traits, genotypic variation among families indicates that continued selection and breeding could produce more desirable genotypes. Somatic chromosome counts on selected genotypes revealed that chromosome transmission between generations appears to be occurring normally. Knowing the ideal environmental conditions could lead to greater success rates in future wide crosses. The greatest maize pollen adhesion and germination on the pistils of Tx3361 (*iap iap*) was observed at 45% humidity. Multiple maize pollen tubes were observed in the sorghum style and ovary at 45% humidity but not at higher humidity

levels. Future interspecific and intergeneric crosses with *iap* sorghum should be performed at low humidity levels. Knowledge of the identity and function of the *iap* allele could lead to discovery of additional genes that regulate barriers to intergeneric hybridization. Fine mapping resolved the location of the *Iap* gene to a 48 kb region on the short arm of chromosome 2. There are three putative genes present in this region, however none of them are easily identified as *Iap*. This research provides valuable insight to proper and efficient utilization of the *iap* allele and a foundation for continued mapping and characterization of *Iap*.

ACKNOWLEDGMENTS

I first want to acknowledge the love and support of my wife, Jessica. Without her encouragement this Ph.D. may not have happened.

I would like to thank Dr. Bill Rooney for giving me the opportunity to get my Ph.D. in his program and for teaching me everything a Northerner needs to know about surviving in Texas. I also want to thank Dr. Patricia Klein for teaching me the ins and outs of molecular genetics and for her patience with my lack of experience with lab equipment. Thank you to Dr. George Hodnett for his frequent assistance with the cytogenetic portion of my project and Natalie Patterson for always being there to help and fix my mistakes. I want to thank Drs. David Stelly and Jorge da Silva for their help and guidance with my research.

Many thanks to the graduate students, student workers, and staff who have assisted me with carrying out my research projects: Matthew Bartek, Leo Hoffman, Dustin Herb, Kyle Burns, Brian Pfeiffer, Francisco Gomez, Geraldo Carvalho, Luke Vacek, Steve Labar, Delroy Collins, Vickie Marriott, Josh Herrington, Robin Jakubik, Michael Klepac, and Kevin Book.

TABLE OF CONTENTS

CHAPTER Page

LIST OF TABLES

TABLE Page

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Sorghum [*Sorghum bicolor* (L.) Moench] is the fifth most widely produced cereal crop in the world with grain production in 2010 of 55.6 million metric tons grown on 40.5 million hectares (Food and Agriculture Organization of the United Nations, 2012). Although statistics are not maintained, it is likely that the area devoted to forage production is even greater. The productivity of sorghum has advanced greatly ever since it was first introduced in the United States (U.S.). This advancement has been due to the creation of a hybrid production system, the large amount of genetic variation available to sorghum breeders, and advancements made in management and production practices. The U.S. Plant Germplasm System contains over 40,000 sorghum accessions with many exotic accessions made available through the sorghum conversion program (Rosenow and Dahlberg, 2000). To utilize accessions belonging to divergent sorghum species, interspecific crosses must be utilized. Due to prezygotic pollen-pistil incompatibilities present in sorghum, many of these crosses are unsuccessful and result in little to no fertilization (Hodnett et al., 2005; Price et al., 2005).

A new technology for interspecific crosses is utilization of the mutant sorghum gene *Inhibition of Alien Pollen* (*iap*). This mutant gene overcomes the fertilization barriers imposed by the wild type form of the gene, allowing pollen tubes of several closely related species and genera to reach the ovary of sorghum that is homozygous for

iap (Bartek et al., 2012; Hodnett et al., 2010; Kuhlman et al., 2008; Laurie and Bennett, 1989; Price et al., 2006). In addition to facilitating hybridization with divergent sorghum species, the use of *iap* has resulted in successful fertilization and hybrid recovery with sugarcane (*Saccharum* spp.), *Erianthus* spp., and *Miscanthus* spp. (Hodnett et al., 2010; G. Hodnett, personal communication, 2011). Pollen tube growth into the ovary has been documented using maize (*Zea mays* L. and subsp. *mexicana*) and buffelgrass (*Pennisetum ciliare*) (Bartek et al., 2012). The *iap* gene has been bred into the released sorghum line Tx3361 to facilitate wide crosses (Kuhlman and Rooney, 2011).

The *iap* allele does not guarantee success in all crosses with sorghum. In an analysis of pollen tube growth on a sorghum line homozygous for *iap*, Bartek et al. (2012) observed that the genotype of the male had a strong influence on the levels of pollen tube growth. Of six accessions from maize (*Zea mays* L.), only four had pollen tubes grow into the sorghum ovary. Kuhlman and Rooney (2011) reported that pollen tube growth to the base of the style in Tx3361 (a sorghum line homozygous for *iap*) was less than that reported by the researchers who first discovered and characterized the allele (Laurie and Bennett, 1989). Kuhlman and Rooney (2011) hypothesized that the variation in pollen tube growth was influenced by environmental conditions. Reduced pollen tube growth was also reported by Kuhlman (2007) when phenotyping maize pollen tube growth on sorghum segregating for *iap*, although no genotypic or environmental influence could be identified as the cause.

The Environmental Protection Agency (EPA) has mandated that the United States must use 36 billion gallons of alternative fuels annually by the year 2022 (United States Congress, 2007). The mandate further requires that 21 billion gallons of that must be from non-cornstarch sources such as sugar and/or cellulose. Sugarcane is known for its high extractable sugar content and the potential to accumulate high cellulosic biomass, although production of sugarcane in the U.S. is not nearly enough to satisfy the demand for alternative fuels. Production in the U.S. is limited to Florida, Louisiana, Texas, and Hawaii with none of the sugarcane being used for ethanol production (Shapouri and Salassi, 2006). Sugarcane is limited to tropical environments due to its perennial growing habit and susceptibility to cold. Commercially grown sugarcane clones today are comprised of as many as five different species of *Saccharum* (Lingle and Tew, 2008). Despite the availability of germplasm from across the world, progress in introgression of traits from unadapted material has been slow or unsuccessful (Berding and Roach, 1987).

Previous attempts to hybridize sorghum and sugarcane were minimally successful (De Wet et al., 1976; Bourne, 1935; Nair, 1999). The frequency of hybrids has been much higher in attempts utilizing sorghum homozygous for the *iap* allele and has led to improvements in the techniques used to produce hybrid plants (Hodnett et al., 2010). This new technology has the potential to allow for rapid and efficient introgression of traits from both sorghum and sugarcane into a new hybrid crop. The drought tolerance of sorghum could potentially be transferred to the hybrid, resulting in a larger growing area with less dependence on frequent watering. The problem of susceptibility to cold can be avoided by creating an earlier maturing hybrid that can be

grown to maturity in a single growing season. In addition, a seeded sugarcane crop could potentially mitigate the high cost currently associated with planting sugarcane. Any or all of these outcomes could potentially increase the growing area of sugarcane in the U.S. and increase production to the levels necessary to meet demand for non-cornstarch based ethanol. No information is currently available on the viability of sorghum × sugarcane hybrids and their feasibility as a unique hybrid crop.

Within that context, the objectives of the current research are:

(1) Identify the optimum relative humidity for intergeneric crosses between sorghum homozygous recessive for *iap* and maize. The results will be used as a starting point to facilitate future crosses between sorghum containing *iap* and other genera.

(2) Characterize sorghum × sugarcane amphidiploids for relevant agronomic traits to determine their viability as a dedicated bioenergy crop. In addition, chromosome numbers in two distinct generations will be analyzed to determine if chromosome transmission from one generation to another is occurring normally or if chromosome abnormalities are occurring.

(3) Fine map the *iap* locus and identify a candidate gene(s) based on phenotype and molecular markers.

CHAPTER II

LITERATURE REVIEW

Interspecific and Intergeneric Hybridization

Interspecific crosses are useful for introducing novel traits into a crop when the desired trait is not naturally present. As biotic and abiotic stresses manifest themselves, the normal solution is to identify resistance in unadapted accessions and breed the trait into an economically viable variety or hybrid. When the desired trait is unavailable in the species of interest, several strategies are available for accessing the trait in a usable form. Genetic transformation has played a key role in introducing alien traits into crops, although the technology is best suited for monogenic or simply inherited traits and for genes that are not present in plants. Another issue with transformation is the intensive testing associated with deregulation. The process can be costly and necessitates that the trait being introduced has an economic benefit large enough to justify the costs associated with introducing it.

Interspecific hybridization is a valuable tool available to plant breeders for introducing variation and novel traits from closely related species. It can be used to introgress both qualitative and quantitative traits of interest. Interspecific crosses have been successfully performed in many crops including tomato (*Solanum lycopersicum* L.), rice (*Oryza sativa* L.), wheat (*Triticum* spp.), and sorghum (*Sorghum* spp.) (De Vicente and Tanksley, 1991; Brar and Khush, 1997; Sharma, 1995; Price et al., 2005).

Successful intergeneric crosses have been reported between wheat (*Triticum timopheevii*) and rye (*Secale cereal*e) (Mujeeb-Kazi, 1981) and between sorghum [*Sorghum bicolor* (L.) Moench] and sugarcane (*Saccharum* spp.) (Hodnett et al., 2010). The success of a wide cross is dependent on many factors including but not limited to genetics, environmental conditions, fertilization barriers, and the direction the cross is made (Sharma, 1995). Hodnett et al. (2005) determined that pollen-pistil incompatibilities were the barrier to interspecific hybridization between *S. bicolor* and divergent *Sorghum* species. The majority of pollen tubes were arrested in the stigma and fertilization was rare. This finding indicates that the first barrier to hybridization is prezygotic and genetic in nature and sorghum accessions would need to be screened in order to find a genetic background that allowed pollen tube growth to the ovary (Hodnett et al., 2005)

Cross-incompatibility in Other Species

Cross-incompatibility genes have previously been identified in other crop species. In wheat (*Triticum aestivum* L.), cross-incompatibility with related species is controlled by genes kr_1-kr_4 and by *SKr* (Alfares et al., 2009). In maize (*Zea mays* L.) several crossability genes have been identified as well. *Teosinte crossing barrier1* (*Tcb1*) prohibits cross-pollination by individuals carrying the *tcb1* gene. The system works reciprocally i.e. male pollen that contains *Tcb1* is rejected on females that carry *tcb1* and females that contain *Tcb1* reject pollen that is carrying *tcb1* (Evans and Kermicle, 2001). In the *Ga1* system in maize, the interactions are different. According to Evans and

Kermicle (2001), if the female is homozygous *Ga1-s* and is pollinated with homozygous *ga1* pollen, no seed will be produced. When a female homozygous for *Ga1-s* is pollinated with a mixture of *Ga1-s* and *ga1* pollen, the *ga1* pollen is rejected. However, when a female homozygous for *ga1* is pollinated with either *Ga1-s* or *ga1* pollen, full seed set results in both cases. To date, no cross-incompatibility genes have been cloned in wheat or maize, although a fine mapping study has been performed for the *SKr* gene in wheat (Alfares et al., 2009).

Interspecific Crosses Utilizing Sorghum

In sorghum, a novel avenue of introgression is through the use of the mutant sorghum gene *Inhibition of Alien Pollen (iap)*. The recessive form of *iap* eliminates fertilization barriers present in the dominant, wild-type form of the gene, allowing pollen tubes of several closely related species and genera to reach the ovary of sorghum (Bartek et al., 2012; Hodnett et al., 2010; Kuhlman et al., 2008; Laurie and Bennett, 1989; Price et al., 2006). The mutant *iap* gene was discovered by Laurie and Bennett (1989) when screening sorghum accessions for the ability to allow maize pollen tube growth in the stigmas. One accession from China, Nr481, allowed maize pollen tube growth into the ovary although no hybrid plants were recovered. Laurie and Bennett (1989) determined the trait was under the control of a single genetic locus, an observation later confirmed by Kuhlman (2007). The *iap* gene was introgressed into sorghum germplasm adapted to the southern U.S. and is present in homozygous form in the released line Tx3361 (Kuhlman and Rooney, 2011).

Price et al. (2006) crossed Nr481 with three divergent sorghum species in an effort to characterize the versatility of the *iap* gene and determine if it could be used to create hybrids where prior efforts were largely unsuccessful (Hodnett et al., 2005; Price et al., 2005). Price et al. (2006) readily obtained vigorous hybrids using *S. angustum* Blake, *S. nitidum* (Vahl) Pers., and *S. macrospermum* Garber as pollinator parents with Nr481 homozygous for *iap*. The use of a sorghum line homozygous for *iap* and embryo rescue resulted in hybrid recovery frequencies of 20.7 and 4.5% for the *S. angustum* and *S. nitidum* crosses, respectively. Embryo rescue was not required for the *S. macrospermum* crosses and the frequency of hybrids was 60% (Price et al., 2006). Kuhlman et al. (2008) produced interspecific hybrids using Nr481 as the female and *S. macrospermum* as the male. The authors analyzed the hybrid plants and determined that chromosomes from *S. macrospermum* ($2n = 40$) were pairing and recombining with the *S. bicolor* chromosomes ($2n = 20$) at meiosis. Additional research utilizing backcross progeny using the same parents showed that at a minimum, portions of the *S. macrospermum* genome had successfully introgressed into the *S. bicolor* genome through recombination, alien chromosome addition, and alien chromosome substitution. Introgression of *S. macrospermum* was as high as 18.6% in one of the BC_2F_1 plants analyzed (Kuhlman et al., 2010).

Benefits of Wide Crosses with Sugarcane

In the U.S., the vast majority of ethanol is produced from agricultural products high in starch, primarily corn or sorghum grain. While this is a critical component of biofuel production, further production from corn is limited by other uses of the grain. If

the entire corn crop in the U.S. was converted to ethanol, roughly 30 billion gallons would be produced, displacing only 25% of the fossil fuels used for transportation (Rooney et al., 2007). An alternative method of ethanol production is to use biomass high in cellulose and hemicellulose, or to use crops such as sugarcane or sweet sorghums with a high soluble sugar content in the stalk.

The EPA has mandated that the U.S. must utilize 36 billion gallons of alternative fuels annually by the year 2022, of which 21 billion gallons must be from non-cornstarch sources such as sugar and/or cellulose (United States Congress, 2007). Sugarcane is widely used for both food sugar and ethanol produced from the sugar or molasses. Brazil has created a successful model for ethanol production from sugarcane with 4.2 billion gallons produced in 2007 on 3 million hectares (Goldemberg, 2007). Because the growing range of sugarcane is limited to tropical environments, production in the contiguous U.S. is limited mainly to the gulf coast region. Currently, sugarcane is produced in four states (Florida, Louisiana, Texas, and Hawaii) with none of the crop being used for ethanol production (Shapouri and Salassi, 2006). The total sugarcane production in the U.S. in 2010 was 24.8 million metric tons of biomass on 355,112 hectares (Food and Agriculture Organization of the United Nations, 2013). Based on a conversion factor yielding 19.5 gallons of ethanol per ton of biomass, the U.S. could produce 533 million gallons of ethanol if the entire crop were used (Shapouri and Salassi, 2006), falling far short of the amount needed to satisfy the requirements set forth by the EPA.

To broaden its adaptation, sugarcane breeders have utilized up to five different species of *Saccharum* (Lingle and Tew, 2008). Despite this work, progress in introgression of germplasm from unadapted material has been slow or unsuccessful (Berding and Roach, 1987). Attempts have been made to hybridize sugarcane with sorghum since they are close relatives that diverged approximately 5 million years ago (Al-Janabi et al., 1994). The hybridization of sorghum and sugarcane into a distinct crop could aid in the pursuit of the standards for ethanol production given the high biomass potential of sugarcane and the drought tolerance of sorghum. A hybrid between the two could potentially be grown on more acres across more diverse environments.

In intergeneric crosses with sugarcane, Hodnett et al. (2010) successfully produced 14,141 seeds in crosses between Tx3361 and several commercial and exotic sugarcane clones. After performing embryo rescue as necessary, 1,371 hybrid plants were grown and screened for relevant agronomic traits. Morphology of these hybrid plants was similar to sugarcane although the panicles were more compact than what is observed in sugarcane (Hodnett et al., 2010). The hybrid plants were male and female sterile and methods are being attempted to mitigate the sterility (G. Hodnett, personal communication, 2012). Previous attempts to hybridize sorghum and sugarcane were minimally successful (De Wet et al., 1976; Bourne, 1935; Nair, 1999). The frequency of hybrids has been much higher in attempts utilizing sorghum homozygous for the *iap* allele and has led to improvements in the techniques used to produce hybrid plants (Hodnett et al., 2010). Premature seed harvest and embryo rescue have led to hybrid seedling recovery rates as high as 33% (Hodnett et al., 2010). The availability of this

new technology coupled with refinement of techniques has the potential to allow for rapid and efficient introgression of traits from both sorghum and sugarcane into a new hybrid crop. The transfer of drought tolerance could result in a larger growing area with less dependence on frequent watering, the problem of susceptibility to cold can potentially be avoided by creating a hybrid that matures in one season, and a seeded sugarcane crop could mitigate the high cost and intensive labor currently required for planting sugarcane. One or all of these potential outcomes could increase the growing area of sugarcane in the U.S.

Preliminary analysis of sorghum × sugarcane F_1 hybrids has been performed (Bartek, 2013). A total of 493 hybrids were planted in a nursery in College Station, TX for observation of agronomic traits. Variation among hybrids was observed for moisture content, brix, juice volume, stalk weight, height, and number of stalks per plant (Bartek, 2013). A replicated trial containing six selected hybrids and an elite sugarcane check revealed that the sugarcane clone was significantly better for juice volume, plot weight, and stalk weight. The sugarcane check was not significantly different from at least one hybrid for brix, leaf weight, number of stalks, height, internode length, and stem diameter (Bartek, 2013). Observation of the plants in the yield trial revealed that there seemed to be variation in phenotype among the plants within a plot and across plots for a given genotype (M. Bartek, personal communication, 2012). It was hypothesized that the variation may have been due to the shedding of chromosomes during vegetative propagation, although this theory has not been tested.

Somaclonal variation from tissue culture has long been recognized and can cause variation in phenotype and genotype in plants derived from the same callus (Larkin and Scowcroft, 1981). Propagation of sugarcane using four different methods was analyzed to determine which of the methods resulted in the fewest number of phenotypic variants (Burner and Grisham, 1995). Callus culture, direct regeneration, shoot tip culture, and bud propagation were compared over two generations using one genotype. Plants derived from bud propagation were 99% normal based on a visual rating while the other three treatments had 2-56% normal plants (Burner and Grisham, 1995).

To realize the full potential of the *iap* gene it must be bred into different sorghum genetic backgrounds depending on the desired end result of the wide cross. For example, an elite sweet sorghum line with high sugar content could potentially result in a sorghum × sugarcane hybrid with a sugar content similar to the original sugarcane parent. Currently, the phenotyping required to track the gene is labor and time intensive and is subject to possible environmental influences (Kuhlman and Rooney, 2011). For marker-assisted selection, a molecular marker with complete linkage to the *iap* gene would be very valuable. Another avenue of investigation would be to fine map and clone the *iap* gene to determine if it shared homology with genes in other crop species. Identification of a previously unidentified homolog in another species could lead to additional intergeneric crosses and introgression of traits between other crops.

Environmental Effects on Cross Compatibility

Kuhlman and Rooney (2011) reported that pollen tube growth to the base of the style in Tx3361 was less than that reported by Laurie and Bennett (1989) for Nr481. Kuhlman and Rooney (2011) hypothesized that the variation in pollen tube growth was influenced by environmental conditions. Reduced maize pollen tube growth on sorghum plants segregating for *iap* was also reported by Kuhlman (2007), although no genotypic or environmental influence could be identified as the cause.

In crosses between *Triticum timopheevii* and *Secale cereale,* Mujeeb-Kazi (1981) observed a difference in embryo recovery between material grown in the greenhouse and growth chamber and concluded that humidity played a large role in the differences. Ballesteros et al. (2003) conducted a study to look at the effect of humidity on the success of haploid production in durum wheat (*Triticum durum*) × maize crosses. Nearly twice as many haploid embryos were produced on average in the low humidity growth chamber (55% humidity in the light, 65% in the dark) as compared to the high humidity growth chamber (65% in the light, 85% in the dark). Sitch and Snape (1987) determined that the frequency of fertilization in crosses made between wheat and *Hordeum bulbosum* was affected by the temperature during pollination and subsequent pollen tube growth. Pollinations were made at 20°C and 26°C using three wheat genotypes and three *H. bulbosum* clones. The mean percent fertilization at 20°C was 24.4 and at 26°C was 11.8. The authors stated that the decreased frequency of fertilization at the higher temperature might be due to reduced stigma receptivity, pollen grain germination, or pollen tube growth.

The effect of environmental conditions on stigma receptivity, pollen viability, and seed set has been studied in detail in several crops. Low humidity, high average temperature, and ample sunshine were found to decrease stigma receptivity and pollen viability in pearl millet [*Pennisetum glaucum* (L.) R.Br.] (Kumar et al., 1995). In a growth chamber experiment on factors affecting seed set in cytoplasmic male sterile wheat, Imrie (1966) determined that a moderate temperature (18°C day, 13°C night) and low humidity (40%-60%) were optimal for maximum seed set. The increased humidity beneath pollinating bags was found to affect seed yield and quality in ryegrass (*Lolium perenne)* crosses (Foster, 1968). Both temperature and humidity were monitored beneath two separate types of pollinating bags placed on ryegrass inflorescences and seed yield and quality were determined. The temperatures under both types of bags were similar to ambient temperatures but the humidity under the bags was much higher than ambient humidity with one type of bag showing a larger increase. The large increase in humidity under the bags was correlated with a decrease in seed yield and quality (Foster, 1968). Further exploration of relevant literature reveals a trend indicating that environmental conditions during flowering and seed maturation have a noticeable effect on stigma receptivity and seed set in sorghum (Jaster, 1985; Moran, 2000; Ross, 1957). However, it must be taken into consideration that the ideal environment for intraspecific crosses may not be the same for interspecific or intergeneric crosses, requiring investigation into the optimum conditions necessary for successful hybridization between species or genera (Pickering, 1980).

CHAPTER III

EFFECT OF HUMIDITY ON INTERGENERIC POLLINATIONS OF *iap* SORGHUM*

Introduction

Interspecific and intergeneric hybridization is an important means to introduce variation and traits into a crop when the trait is not present within the primary gene pool or traditional methods are incapable of producing the desired results. Genetic transformation has also been useful to introduce traits not present in the species of interest. However, transformation is best suited for monogenic or simply inherited traits.

It is possible to produce interspecific and intergeneric hybrids when the genotypes of each species are compatible enough to produce viable hybrid seed. The success rate of wide crosses is highly dependent on the genotypes used and environmental conditions during crossing and seed development (Ballesteros et al., 2003; Campbell et al., 2001; Mujeeb-Kazi 1981; Sitch and Snape, 1987).

Pollination begins when pollen grains land on the stigma. At this point lipids, carbohydrates, and proteins from the pollen grain and the stigma mix and compatibility

*Reprinted with permission from "Effect of humidity on intergeneric pollinations of *iap* (*Inhibition of Alien Pollen*) sorghum [*Sorghum bicolor* (L.) Moench]" by John R. Gill, William L. Rooney, Patricia E. Klein, 2014. *Euphytica*, DOI 10.1007/s10681-014-1113-5. Copyright 2014 by Springer Science + Business Media B.V.

determinations are made (Swanson et al., 2004). Wet stigmas are covered with a liquid secretion that enables adhesion of the pollen grain regardless of the compatibility of the genotypes (Heslop-Harrison 1975; Swanson et al., 2004). Dry stigmas do not guarantee the adhesion of pollen grains, and signaling must occur between the pollen and the stigma before hydration and germination proceed. The signaling system has been partially determined in *Brassica*: the pollen coat contains a *S*-locus cysteine-rich protein (SCR) that determines the compatibility of the male genotype with that of the female, and the stigma contains a *S*-locus receptor kinase (SRK) that determines the compatibility of the female genotype with that of the male (Swanson et al, 2004).

The barrier to interspecific hybridization between *S. bicolor* and other sorghum species was determined to be due to pollen-pistil incompatibilities (Hodnett et al., 2005). The discovery of the *Inhibition of Alien Pollen* (*iap*) allele in sorghum was critical for allowing pollen tubes of several closely related species and genera to reach the ovary of sorghum (Bartek et al., 2012; Hodnett et al., 2010; Kuhlman et al., 2008; Laurie and Bennett, 1989; Price et al., 2006). The gene, when homozygous in the recessive form, overcomes the fertilization barrier that has previously resulted in limited success in hybridization of sorghum with other genera (De Wet et al., 1976; Bourne 1935; Nair 1999).

The *iap* allele does not guarantee success in all crosses with sorghum. In an analysis of pollen tube growth on a sorghum line homozygous for *iap*, Bartek et al. (2012) observed that the genotype of the male had a strong influence on the levels of pollen tube growth. Of six accessions from maize (*Zea mays* L.), only four had pollen

tubes grow into the sorghum ovary. Kuhlman and Rooney (2011) reported that pollen tube growth to the base of the style in Tx3361 (a sorghum line homozygous for *iap*) was less than that reported by the researchers who first discovered and characterized the allele (Laurie and Bennett, 1989). Kuhlman and Rooney (2011) hypothesized that the variation in pollen tube growth was influenced by environmental conditions. Reduced pollen tube growth was also reported by Kuhlman (2007) when phenotyping maize pollen tube growth on sorghum segregating for *iap*, although no genotypic or environmental influence could be identified as the cause.

The effect of environmental conditions on stigma receptivity, pollen viability, and seed set has been studied in detail in several crops. Low humidity, high average temperature, and ample sunshine were found to decrease stigma receptivity and pollen viability in pearl millet [*Pennisetum glaucum* (L.) R.Br.] (Kumar et al., 1995). In a growth chamber experiment on factors affecting seed set in cytoplasmic male sterile wheat, Imrie (1966) determined that a moderate temperature (18°C day, 13°C night) and low humidity (40%-60%) were optimal for maximum seed set. The increased humidity beneath pollinating bags was found to reduce seed yield and quality in ryegrass (*Lolium perenne)* crosses (Foster 1968). Further exploration of relevant literature reveals a trend indicating that environmental conditions during flowering and seed maturation have a noticeable effect on stigma receptivity and seed set in sorghum (Jaster, 1985; Moran, 2000; Ross, 1957).

Environmental conditions influence intergeneric crosses as well. In crosses between *Triticum timopheevii* and *Secale cereale*, Mujeeb-Kazi (1981) observed a

difference in embryo recovery between material grown in the greenhouse and growth chamber and concluded that humidity played a large role in the differences. Ballesteros et al. (2003) conducted a study to look at the effect of humidity on the success of haploid production in durum wheat (*Triticum durum*) × maize crosses. Nearly twice as many haploid embryos were produced on average in the low humidity growth chamber as compared to the high humidity growth chamber. Sitch and Snape (1987) determined that the frequency of fertilization in crosses made between wheat and *Hordeum bulbosum* was affected by the temperature during pollination and subsequent pollen tube growth.

Given both the genetic and environmental influences, it is likely that the ideal environment for intraspecific crosses may not be the same for interspecific or intergeneric crosses. Thus, investigation into the optimum conditions necessary for successful hybridization between species or genera is usually required (Pickering, 1980). The objective of this research was to identify the optimum relative humidity for intergeneric crosses between sorghum homozygous recessive for *iap* and maize. This information is critical to identify optimum conditions to facilitate future crosses between sorghum containing *iap* and other genera.

Materials and Methods

Plant Material

To phenotype pollen adhesion and pollen tube growth, the sweet corn hybrid ‘Kandy Korn’ (River City Seed Co., Little Rock, AR) was used as the pollinator since it

had the highest incidence of pollen tube growth into the ovary of the six maize accessions tested (Bartek et al., 2012). Two sorghum genotypes were used as female parents: ATx623 (*Iap Iap*) and Tx3361 (*iap iap*). All three genotypes were planted each week for seven weeks to ensure a nick in flowering between the maize and sorghum. Due to space limitation in the growth chambers, these plantings and initial growth were conducted in the greenhouse. The pots were planted at six seeds per pot and were thinned to three plants per pot after two weeks of growth. The lighting and temperature schedules for both chambers were as follows: 6 a.m. lights on ⅔, temperature 22°C; 6:30 a.m. lights on full, temperature 31°C; 7:30 p.m. lights on ⅔, temperature 31°C; 8:00 p.m. lights off, temperature 22°C. Sorghum plants were moved into the chambers when they reached the boot stage of growth (Growth stage 5, Vanderlip and Reeves, 1972) and maize plants were moved in when they reached the end of the vegetative stage of growth and the tassel was barely visible deep in the whorl. A HOBO U10 data logger (Onset, Bourne, MA.) capable of recording both temperature and humidity was placed in each growth chamber to ensure that conditions did not deviate from the set points during the duration of the study.

Pollinations were made using male and female within three different humidity levels: 45%, 65% and 85%. In addition, pollinations were made between the female at 85% and male at 65% (85% × 65%), and the female at 65% and male at 85% (65% × 85%). At least one pollination of each treatment was made onto both Tx3361 and ATx623.

To verify that the sorghum stigmas were receptive in all environments, a male-fertile Tx3361 panicle was used to pollinate a male-sterile Tx3361 panicle in each growth chamber and seed development was assessed. To confirm that the maize pollen was viable under each set of conditions, maize pollen was dusted onto silks on the same plant and seed development was assessed. These controls confirmed that both pollen and ovules were viable.

For consistent quantities of pollen for each pollination, a scoop approximately 100 μl in volume was utilized for each pollination. To ensure only viable pollen grains were available each day for pollinations, the maize tassels were stripped of all anthers and shaken to remove any residual pollen at the end of each day. Once the male-sterile sorghum panicle had completely flowered, excess florets were removed until approximately 40 were remaining. Pollen was collected by tapping the maize tassels over a large glass petri dish followed by collection of the pollen in the scoop. This scoop of pollen was transferred to a new glass petri dish and the pollen was gently dusted over the sorghum stigmas using a small horsehair paintbrush.

Phenotyping

Twenty-four hours after pollination, pollinated florets were harvested and placed into glass vials containing 3:1 (95% ethanol : glacial acetic acid) fixative for a minimum of one week. The pistils were extracted from the florets and put in 70% ethanol at -20°C until needed. The pistils were processed according to the protocol described by Kho and Baer (1968) with modifications as described by Hodnett et al. (2005). Slides were

analyzed with a Zeiss Universal II microscope (Carl Zeiss Inc., Gottingen, Germany) with a 10X Neofluor objective. Fluorescence of the callose in the pollen tubes was induced using 390-420 nm light emitted from a mercury lamp with a 450 nm emission filter. Twenty-four pistils were analyzed from each pollination and pollen counts were taken for each of the following stages: adhered, germinated, pollen tube in stigma axis, pollen tube in style, and pollen tube in ovary.

Statistical Analysis

Data were analyzed using the mixed model procedure of SAS version 9.3 (SAS Institute, 2011). Females and treatments were considered fixed effects and replications within treatments and females were considered random effects. Individual pistils analyzed for each female by treatment combination were considered replications.

Results

Control pollinations (Tx3361/Tx3361 and Kandy Korn/Kandy Korn) resulted in satisfactory seed set, indicating that the maize pollen and sorghum stigmas were viable at all humidities tested. Mean pollen adhesion and germination were greater for Tx3361 than ATx623 at each humidity level (Table 1) and there was a significant difference between Tx3361 and ATx623 for the 45% and 65% × 85% humidity levels (Figure 1). There were significant differences in pollen adhesion and germination between humidity levels for both Tx3361 and ATx623.

Table 1. Mean and standard deviation for each of the five humidity treatments on both sorghum lines.

Treatment†	45%		65%		85%		65% × 85%		85% × 65%	
ATx623	mean	std dev‡	mean	std dev	mean	std dev	mean	std dev	mean	std dev
adhesion§	0.7c¶	1.0	8.0a	6.6	5.5a	4.1	1.9bc	2.4	4.9ab	4.0
germination	0.6c	0.9	7.8a	6.6	5.0ab	3.9	1.8bc	2.4	4.5b	3.8
stigma axis	0a	0	0a	0	0a	0	0a	0	0a	0
style	0a	0	0a	0	0a	0	0a	0	0a	0
ovary	0a	0	0a	0	0a	0	0a	0	0a	0
Tx3361										
adhesion	78.0a	27.5	13.0b	6.0	6.9b	7.8	12.9b	8.1	8.9b	9.7
germination	76.6a	27.1	10.0b	5.0	6.5b	7.2	11.7b	7.3	8.4b	9.1
stigma axis	1.8a	2.1	0.5b	0.8	0.1b	0.3	0.1b	0.4	0.1b	0.2
style	0.1a	0.2	0a	0	0a	0	0a	0	0a	0
ovary	0.1a	0.2	0a	0	0a	0	0a	0	0a	0

†Humidity in the growth chamber at the time of pollination. 65% × 85% indicates that the female was at 65% and the male was at 85%, 85% × 65% indicates that the female was at 85% and the male was at 65%.

‡Standard deviation

§Mean number of pollen grains or pollen tubes at each position on the pistil

¶Means within a row followed by the same letter were not significantly different at the 0.05 probability level based on Tukey's honestly significant difference (Tukey, 1949).

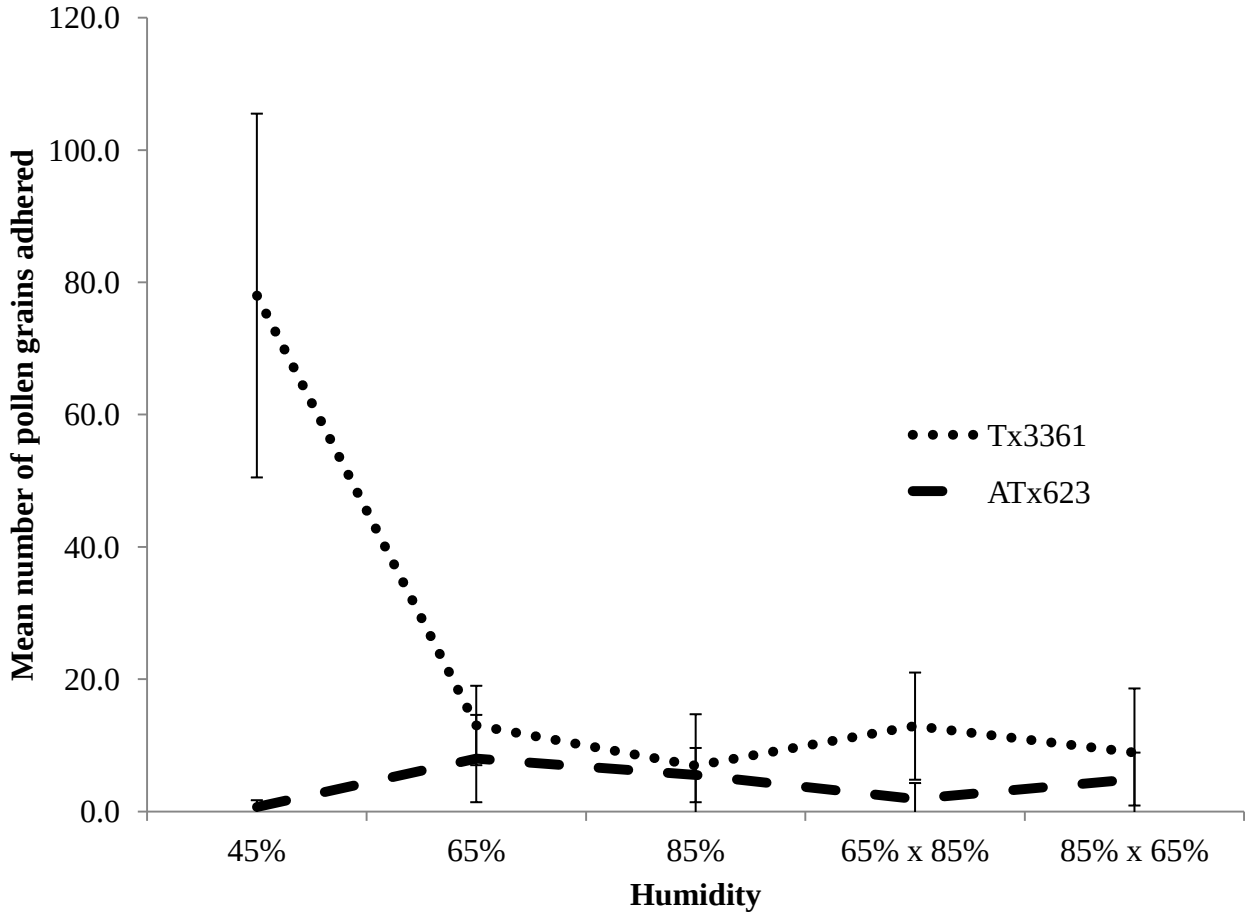

Figure 1. Mean pollen adhesion across humidity levels and females. Error bars indicate the standard error of the calculated mean.

In pollinations onto ATx623, very low pollen adhesion was observed on the pistils for all humidity levels (Table 1). Pollen adhesion and germination on ATx623 was highest at 65% humidity and significantly lower at 45% humidity. Pollen tube growth into the stigma axis, style, or ovary was not observed in any of the treatments with ATx623.

In pollinations onto Tx3361, mean pollen adhesion and germination increased as the humidity decreased, indicating that low humidity levels favor success of intergeneric pollinations utilizing the *iap* allele in the Tx3361 genetic background (Figure 1). In fact, pollen adhesion on Tx3361 was six-fold greater at 45% humidity than at 65% humidity. A single pollen tube was observed in the style and ovary on one pistil out of 24 analyzed for the 45% treatment. In addition, several pollen tubes were observed in the stigma axis of Tx3361 for each of the five treatments.

Discussion

The significant difference between ATx623 and Tx3361 for both pollen adhesion and germination confirms that *iap* strongly influences both of these traits. Of the humidity levels tested, the lower humidities were more favorable for adhesion and germination on Tx3361.

The large difference in the mean adhesion and germination between the 65% and the 65% × 85% treatments on ATx623 implies that the environment of pollen formation affects the success of the pollination more than the environment of ovule development. In contrast, the mean adhesion and germination of the 85% × 65% treatment is similar to

that of the 85% treatment. It is possible that the humidity of 85% affected the pollen sourced from the 65% environment before it was able to adhere to the stigma.

On a species such as sorghum with dry stigmas, the pollen grain must be compatible for hydration to occur (Swanson et al., 2004). Therefore, the maize pollen would not be expected to hydrate and germinate under any of the environmental conditions imposed by this experiment. Given that pollen germination on ATx623 was lowest at 45% humidity and increased with higher humidities, it may be that the maize pollen is hydrating independently of signals from the stigma due to the high humidity at 65% and 85%.

The lack of pollen tube growth to the stigma axis on ATx623 is consistent with the findings of Bartek et al. (2012) in which the authors observed poor pollen adhesion and germination using the same genotypes. This was expected given the intergeneric nature of the cross and the lack of the *iap* allele in homozygous form in ATx623.

The low incidence of pollen tube growth to the style and ovary obtained under these five treatments on Tx3361 is not typical of that observed in previous studies (Kuhlman and Rooney, 2011; Bartek et al., 2012). Pollen tube growth at least to the base of the style was the criteria used to select for the *iap* allele in a segregating population, leading to the expectation that we would observe more pollen tubes in the style using these treatments (Kuhlman and Rooney, 2011). It is possible that lower humidities would further increase pollen tube germination and growth, but those levels were not tested in this study.

The mean pollen adhesion and germination on Tx3361 at 65% humidity was similar to that observed with the 65% × 85% treatment, indicating that the female in the cross is the determining factor in the success of the pollination when the female is homozygous for *iap*. This hypothesis is supported by the similarity in the mean adhesion and germination between the 85% treatment and the 85% × 65% treatment. This was not consistent with the results from ATx623 and is likely due to the presence of the *iap* allele in homozygous form in Tx3361.

Previous intergeneric crosses utilizing sorghum homozygous for *iap* have been met with varied success (Hodnett et al., 2010; Bartek et al., 2012). Hybrids with sugarcane (*Saccharum* spp.) were readily obtained by Hodnett et al. (2010) under ambient conditions in a sugarcane crossing house. Bartek et al. (2012) obtained varying success with 16 accessions used as pollinator parents under ambient conditions in a standard greenhouse. Seven accessions showed pollen tube growth to the ovary on Tx3361, indicating that the male used as the pollinator had a large effect on pollen tube growth.

Under ambient conditions in a greenhouse, humidity levels can vary due to multiple factors. Results herein demonstrate that a lower humidity is desirable for obtaining maize pollen adhesion and germination on sorghum stigmas. By extrapolation, a lower humidity that allows for greater adhesion should allow for greater pollen tube growth and therefore, more opportunity for fertilization should the two genera be sufficiently compatible. It would be beneficial for future intergeneric crosses utilizing

sorghum homozygous for *iap* to take place in an environment where the humidity is naturally low during an extended period of time.

It is possible that vapor pressure deficit, which is a combination of temperature and humidity, could clarify the role of environment on the success of fertilization. The focus of this study was on humidity, but other studies have indicated that temperature has a large effect on fertilization and seed set in intraspecific crosses as well (Prasad et al., 2006; Prasad et al., 2008; Baker et al., 1992). A detailed study may discover that both temperature and humidity play important roles in the success of fertilization.

The pollen-pistil incompatibility observed by Hodnett et al. (2005) using divergent sorghum species is identical to that observed in crosses between ATx623 and maize, manifesting itself in the form of aberrant pollen tube growth. The presence of *iap* in homozygous form in Tx3361 reduces this barrier, allowing pollen grains to adhere, germinate, and grow towards the ovary. It is likely that the *Iap* allele is at least partially in control of this signaling process in the female. It is possible that the *iap* allele is the result of a mutation that renders the wild type gene non-functional, resulting in the lack of a barrier to hydration of alien pollen.

Sorghum and sugarcane diverged approximately 5 million years ago (Al-Janabi et al., 1994), while sorghum and maize diverged approximately 11-28 million years ago (Paterson et al., 2004). It is likely that the *iap* allele overcomes the incompatibility barrier present in the stigmas that evolved before the divergence of the sorghum/sugarcane ancestor from maize and that other barriers developed after the

divergence of the sorghum/sugarcane ancestor from maize. To obtain hybrids with maize, those barriers must be identified and modified to facilitate fertilization.

The results obtained in this research provide insight into optimum conditions to facilitate wide hybridization in sorghum. Low humidity coupled with temperatures optimal for sorghum resulted in increased maize pollen adhesion and germination on the stigmas of sorghum homozygous for *iap*. Future research should focus on identification of the *Iap* gene and how it functions in determining pollen-pistil incompatibility.

CHAPTER IV

AGRONOMIC AND CYTOGENETIC EVALUATION OF AMPHIDIPLOIDS

Introduction

Demand for fuels across the world has led to increased fuel prices and fostered renewed interest in renewable sources of alternative energy. The EPA has mandated that the U.S. utilize 36 billion gallons of alternative fuels annually by the year 2022, of which 21 billion gallons must be from non-cornstarch sources such as sugar and/or cellulose (United States Congress, 2007). Sugarcane (*Saccharum officinarum* L.) is widely used for both food sugar and ethanol produced from the sugar or molasses. Brazil has created a successful model for ethanol production from sugarcane with 4.2 billion gallons produced in 2007 on 3 million hectares (Goldemberg, 2007). Because sugarcane production is limited to tropical environments, it can only be produced in a few select areas in the U.S. Currently, sugarcane is produced in four states (Florida, Louisiana, Texas, and Hawaii) with none of the crop being used for ethanol production (Shapouri and Salassi, 2006). The total sugarcane production in the U.S. in 2010 was 24.8 million metric tons of biomass on 355,112 hectares (Food and Agriculture Organization of the United Nations, 2013). Based on a conversion factor yielding 19.5 gallons of ethanol per ton of harvested biomass, the U.S. could produce 533 million gallons of ethanol if the entire crop was used (Shapouri and Salassi, 2006), falling far short of the amount needed to satisfy the requirements set forth by the EPA.

Until recently, attempts to hybridize sugarcane with sorghum [*Sorghum bicolor* (L.) Moench] had limited success even though the two species are close relatives that diverged approximately 5 million years ago (Al-Janabi et al., 1994). Intergeneric hybridization of sorghum and sugarcane could be useful for introgression as well as the development of a new crop. This biofuel crop potentially combines the high biomass potential of sugarcane with the drought tolerance of sorghum, allowing production in a wider range of areas.

Initial attempts at hybridization of sorghum and sugarcane were minimally successful (De Wet et al., 1976; Bourne, 1935; Nair, 1999). A mutant gene named *Inhibition of Alien Pollen* (*iap*) has been identified that allows pollen tubes of several closely related species and genera to reach the ovary of sorghum (Bartek et al., 2012; Hodnett et al., 2010; Kuhlman et al., 2008; Laurie and Bennett, 1989; Price et al., 2006). The gene was first identified by Laurie and Bennett (1989) in the sorghum accession Nr481 from China for its ability to allow maize pollen tube growth to the sorghum ovary, although no hybrid plants were recovered. They determined the trait was under the control of a single genetic locus, an observation later confirmed by Kuhlman (2007). The *iap* gene was introgressed into sorghum germplasm adapted to the southern U.S. and is present in homozygous form in the released line Tx3361 (Kuhlman and Rooney, 2011). Hodnett et al. (2010) crossed Tx3361 with several sugarcane clones and produced 14,141 hybrid seeds. After performing embryo rescue, the authors obtained 1,371 hybrid plants that were subsequently screened for agronomic desirability. The hybrid plants have a morphology similar to sugarcane although the panicles are more

compact and are male and female sterile (Hodnett et al., 2010). Amphidiploids were created by doubling the intergeneric F_1's with colchicine in an attempt to mitigate the sterility issues. The presence of *iap* has greatly increased the success of both interspecific and intergeneric hybrid recovery. Furthermore, the process has been improved. For example, premature seed harvest and embryo rescue have resulted in sorghum × sugarcane seedling recovery rates as high as 33% (Hodnett et al., 2010).

The availability of this new technology coupled with the refinement of techniques has the potential to allow for rapid and efficient introgression of traits from both sorghum and sugarcane into a new hybrid crop. The transfer of drought tolerance could result in a wider range of adaptation and reduced dependence on irrigation. In addition, if seed set could be improved, there remains the possibility of a seed-planted intergeneric sugarcane crop, which would mitigate the high cost and intensive labor currently required for planting sugarcane. Any of these potential outcomes could increase the growing area of sugarcane in the U.S. and create enough production to help meet the requirements for non-cornstarch based ethanol.

Preliminary analysis of sorghum × sugarcane intergeneric F_1 hybrids has been performed (Bartek, 2013). A total of 493 hybrids were planted in a nursery in College Station, TX for observation of agronomic traits. Variation among hybrids was observed for moisture content, brix, juice volume, stalk weight, height, and number of stalks per plant (Bartek, 2013). A replicated trial planted in Weslaco, TX in 2010 containing six selected hybrids and an elite sugarcane check revealed that the sugarcane clone was significantly better for juice volume, plot weight, and stalk weight. The sugarcane check

was not significantly different from at least one hybrid for brix, leaf weight, number of stalks, height, internode length, and stem diameter (Bartek, 2013). Observation of the plants in the yield trial revealed that there seemed to be variation in phenotype among the plants within a plot and across plots for a given genotype (M. Bartek, personal communication, 2012). It was hypothesized that the variation may have been due to the shedding of chromosomes during vegetative propagation, although this theory was not confirmed.

Somaclonal variation from tissue culture has long been recognized and can cause variation in phenotype and genotype in plants derived from the same callus (Larkin and Scowcroft, 1981). Propagation of sugarcane using four different methods was analyzed to determine which of the methods resulted in the fewest number of phenotypic variants (Burner and Grisham, 1995). Callus culture, direct regeneration, shoot tip culture, and bud propagation were compared over two generations using one genotype. Plants derived from bud propagation were 99% normal based on a visual rating while the other three treatments had 2-56% normal plants (Burner and Grisham, 1995).

The objectives of this research were to characterize sorghum × sugarcane amphidiploids for relevant agronomic traits to determine their viability as a dedicated bioenergy crop. Agronomic data from the amphidiploids were compared to that of the original intergeneric F_1 hybrids to determine if doubling the genome had any effect on productivity and/or usefulness as a bioenergy crop. In addition, chromosome numbers in two distinct generations were analyzed to determine if chromosome transmission from one generation to another is occurring normally or if chromosome abnormalities such as

aneuploidy are occurring. The phenotypes of the two generations were compared to determine if changes in appearance and agronomic performance were occurring due to somaclonal variation.

Materials and Methods

Plant Material

Creation of the intergeneric F_1 seed is described in detail by Hodnett et al. (2010). Confirmed intergeneric F_1 hybrids between sorghum and sugarcane were planted in a space plant nursery in College Station, TX in 2009. Superior genotypes were selected based on agronomic characteristics and were subjected to photoperiod treatment in the sugarcane crossing house in Weslaco, TX in an attempt to induce flowering. Several of the genotypes entered the reproductive phase, but were male and female sterile. Sterility is likely a result of the plants having a haploid genome from each parent; consequently, chromosomes from each genome do not pair normally at meiosis and non-functional gametes form.

To mitigate this problem, several selected genotypes underwent chromosome doubling using colchicine. Each plant derived from a colchicine treated callus was considered a distinct genotype due to the phenotypic variability among clones that can occur with the use of tissue culture (reviewed by Larkin and Scowcroft, 1981). The amphidiploid nature of the colchicine treated plants was verified using an Accuri C6 flow cytometer (BD Biosciences, San Jose, CA). Eighteen of these genotypes from three separate families were selected for further analysis and characterization (Table 2).

Table 2. Amphidiploid genotypes, the F_1 they resulted from, and the sugarcane parents.

Sugarcane Parent	F_1 Hybrid	Generation One	Generation Two
4156	L07-9s	11BAD1	12BAD41
4156	L07-9s	11BAD2	12BAD42
4156	L07-9s	11BAD3	12BAD43
4156	L07-9s	11BAD5	12BAD44
4156	L07-9s	11BAD6	12BAD45
4156	L07-9s	11BAD7	12BAD46
4156	L07-9s	11BAD8	12BAD47
4156	L07-9s	11BAD9	12BAD48
4156	L07-9s	11BAD10	12BAD49
4156	L07-9s	11BAD11	12BAD50
4156	L07-9s	11BAD13	12BAD51
4156	L07-9s	11BAD15	12BAD52
4156	L07-9s	11BAD16	12BAD53
1638	09BSX4221	11BAD22	12BAD54
1638	09BSX4221	11BAD23	12BAD55
2374	09BSX5115	11BAD30	12BAD57
2374	09BSX5115	11BAD34	12BAD58
2374	09BSX5115	11BAD36	12BAD59

To determine if somaclonal variation was occurring during vegetative propagation, two distinct generations were created in the greenhouse. The eighteen selected genotypes were vegetatively propagated from the original plants that had been doubled. This material is considered generation one for the purposes of this reserach and each genotype name is preceded by '11' to indicate that it was propagated in 2011 (Table 2). Once sufficient vegetative growth had occurred, the generation one material was again vegetatively propagated to form generation two. The name of each generation two genotype is preceded by '12' to indicate that it was propagated in 2012 (Table 2). Several pots of each intergeneric F_1 parent were planted as well.

The material was increased in the greenhouse to produce enough biomass for field trials. Cane was harvested from the greenhouse on September 23-24, 2012. Each plant was cut at the base of the stalk, stripped of its leaves, cut into three-foot long billets, and packaged in Versi-Dry lab soakers (Nalgene, Rochester, NY) saturated with a 0.2% solution of BanRot 40WP (Scotts, Marysville, OH). The eighteen genotypes of both generation one and generation two, the three intergeneric F_1 parents, and three elite sugarcane checks were planted in a randomized complete block design with two replications at the Texas A&M University research farm at Weslaco, TX on September 25, 2012. The soil type at the site is a Hidalgo sandy clay loam (fine-loamy, mixed, active, hyperthermic Typic Calciustolls). Plots were six meters in length with a 1.5 meter alley and 1.5 meters between each plot. Billets were planted approximately 25 centimeters deep in a raised bed with the billets overlapping each other by approximately two nodes at each end. Irrigation was applied through a combination of sub-surface drip

tape and flood irrigation and was not limiting throughout the growth of the yield trial. Plots were treated with Aatrex (Syngenta, Greensboro, NC) and Sencor (Bayer Crop Science, Germany) for the control of weeds as needed. Fertilizer was applied on January 15, 2013 with 15-15-15 (N-P_2O_5-K_2O) spread at a rate of 275 grams per plot.

Harvest and Data Collection

Approximately 15 months after planting (December 10, 2013), two representative plants were harvested from each plot for processing. The plants were weighed, stripped of their leaves, and weighed again to obtain the proportion of plot weight attributable to stalks. The stalks were passed through a Moenda Cana Shop 200 sugarcane mill (Vencedora Maqtron, Brazil) twice in order to obtain a juice sample. The concentration of soluble solids in the juice was measured in percent brix (1% brix is 1 g sucrose in 100 g solution) using a digital refractometer (Atago, Bellevue, WA). A 15 mL sample of juice was collected and kept on ice until placed in the freezer. At extraction, 2 ml of 8% sodium azide (GFS Chemicals, Inc., Columbus, OH) was added to the juice sample to prevent sugar degradation. In addition to the sampling, data were collected on a plot basis for plant height, lodging, stand density, and uniformity of plants. Height was measured in centimeters (cm) from the base of the plant to the growing point. Lodging was measured on a percentage basis with a higher percentage indicating more severe lodging; stand density was evaluated on a scale from 1-5 with 1 being a dense stand and 5 meaning no plants; uniformity was evaluated on a scale from 1-4 with 1

being very uniform and 4 indicating a significant amount of height variation within the plot.

Biomass yield was estimated by harvesting the plots on December 16-17, 2013 using a single row New Holland 707 forage harvester attached to a John Deere 5410 tractor with an incorporated weigh bucket system utilizing a single load cell. Entire plots were harvested and plot weights recorded. A biomass sample was collected from each plot, weighed, dried in a forced air oven for a minimum of 72 hours, and weighed again to obtain the moisture content of the harvested biomass. Juice weight was calculated as the difference in the wet weight and dry weight of the plot. Total fermentable sugar yields were calculated using the equation developed by Corn (2009), where fermentable sugar yield = 0.95 × juice weight × 0.97 × 0.873 × (% Brix/100). Although not all soluble sugars are extracted, 95% extraction efficiency is assumed when modern techniques are utilized (Bennett and Anex, 2009). The juice extracted using a roller press has been shown to be slightly over representative of the actual brix concentration of the entirety of the juice contained in the stalk, so a constant of 0.97 is applied to correct for this bias (Engelke, 2005). The brix reading evaluates all soluble solids contained in the juice, only a portion of the solids are actually fermentable sugar. Corn (2009) used High Performance Liquid Chromatography (HPLC) to determine that fermentable sugars accounted for approximately 0.873 of the soluble solids in solution.

Dry biomass samples were ground in a Wiley Mill (Arthur H. Thomas Co., Philadelphia, PA) to a particle size small enough to pass through a 2 mm screen. Each biomass sample and juice sample was analyzed on a FOSS near-infrared XDS Rapid

Content Analyzer (FOSS NIRSystems, Inc., Laurel, MD). Spectrum data for biomass samples were converted into composition data using a calibration curve developed by the Texas A&M University sorghum quality lab and the National Renewable Energy Laboratory (Wolfrum et al., 2013). Spectrum data for the juice samples were converted using a calibration curve developed by the same entities. Biomass samples were analyzed for ash, protein, sucrose, lignin, glucan, xylan, galactan, arabinan, and cellulose composition and juice samples were analyzed for sucrose, glucose, and fructose composition.

Chromosome Counts

Chromosome counts were obtained from a subset of the genotypes in both generations to determine if chromosome numbers were stable in these progeny. Actively growing root tips were harvested into a saturated aqueous solution of α-bromonapthalene for three hours kept at room temperature in the dark. Root tips were taken from only one plant from each genotype in each generation. The α-bromonapthalene was removed and samples were fixed in 3:1 ETOH/glacial acetic acid for a minimum of 24 hours. Root tips were washed with water three times for 20 minutes each time to remove the fixative. The terminal 1 mm of each root tip was removed and placed in a 0.5 ml epitube filled with 0.2M HCl. The samples were hydrolyzed in a 37°C water bath for 30 minutes. The HCl was removed and samples were rinsed with water for 10 minutes. The water was removed and 100 µl of a solution of 15% pectinase (from *Aspergillus aculeatus*) and 35% cellulase (from *Trichoderma*

reesei) (Sigma Aldrich, St. Louis, MO) in 2 mM citrate buffer was added to the root tips. The samples were denatured in a 37°C water bath for 45 minutes to 2.5 hours, with larger root tips staying in longer. After denaturation, the enzymes were removed and the samples were rinsed twice with water. Root tips were placed on a glass slide with one drop of 3:1 ETOH/glacial acetic acid. The root tip was immediately macerated with tweezers in order to spread the cells around. After air-drying, slides were stained with Azure Blue, dried, and fixed with Permount. Slides were observed using a Zeiss Universal II microscope (Carl Zeiss Inc., Gottingen, Germany). A minimum of eight quality spreads of condensed chromosomes were used to determine the somatic chromosome number of each selected genotype in each generation. Determination of chromosome numbers was made by taking into account the mean and mode for all the spreads in addition to the recorded chromosome number from the highest quality spread obtained for each genotype.

Statistical Analysis

All analyses of variance and multiple comparison procedure tests were performed using the general linear model procedure of SAS version 9.3 (SAS Institute 2011). Replications were considered a random effect and genotypes were considered a fixed effect.

Results and Discussion

Chromosome Counts

Analysis of the somatic chromosome numbers of a selected group of genotypes revealed that chromosome numbers were stable within a genotype and across generations (Table 3). No significant deviation from expected chromosome number was observed between the two generations. Chromosome numbers varied somewhat from generation one to generation two, although given the large range in chromosome numbers observed across multiple cells, this may be due to breakage/loss of the chromosomes during preparation of the spreads (Table 3). The breakage of a chromosome would make it appear as if there were two chromosomes instead of one, leading to an inflated chromosome count. Chromosomes can become scattered on the slide when the cell membrane breaks before the cell is fixed to the slide surface. Currents in the fixative then have the chance to move chromosomes far away from the central group of chromosomes (Spurbeck et al., 1996). This situation would result in fewer chromosomes being counted than are actually present. Considering the intricacies involved in the preparation of chromosome spreads, there is no evidence to believe that chromosome transmission is occurring abnormally. It appears that complete doubling of the chromosomes may not have occurred with all of the amphidiploids after treatment of the intergeneric F_1 with colchicine (Table 3). The expected chromosome number of 11BAD23 was 136 based on a chromosome number of 68 for the intergeneric F_1 09BSX4221, although only approximately 130 chromosomes were observed for 11BAD23. This may be a result of incomplete doubling/chromosome loss following

Table 3. Chromosome counts of selected amphidiploid genotype pairs and the F_1 hybrids they resulted from.

Genotype	Generation†	Chromosomes	Mean	Mode	Spreads Observed	Range
L07-9s	F_1	62	62	62	8	60-64
11BAD13	Gen 1	118	121	118	8	118-128
12BAD51	Gen 2	126	127	126	11	123-133
09BSX4221	F_1	68	68	68	8	66-68
11BAD22	Gen 1	136	125	136	9	109-136
12BAD54	Gen 2	130	130	132	9	120-136
09BSX4221	F_1	68	68	68	8	66-68
11BAD23	Gen 1	130	130	132	10	124-137
12BAD55	Gen 2	134	134	134	8	132-137
09BSX5115	F_1	60	59	60	8	56-62
11BAD34	Gen 1	120	121	122	9	111-130
12BAD58	Gen 2	120	121	120	9	118-124

†Gen 1, generation 1; Gen 2, generation 2

treatment with colchicine, or it may be a result of inaccurate chromosome numbers obtained for 11BAD23. Chromosome numbers behaved as expected for 09BSX5115, 11BAD34, and 12BAD58 (Table 3). This may result from the genotypes being particularly amenable to the chromosome count protocol in use, or it may be due to the reduced number of chromosomes present in the intergeneric F_1, resulting in fewer opportunities for chromosome breakage, loss, or undetected overlap.

It was not possible to definitively distinguish between the sorghum and sugarcane chromosomes. Thus, it was not possible with the methods used to determine if the amphidiploids had retained the complete diploid set of sorghum chromosomes (Figure 2). Slight differences in chromosomal condensation patterns between sorghum and sugarcane chromosomes may prevent distinguishing between the two species based on size. Additional research using cytological techniques such as genomic *in situ* hybridization (GISH) may be necessary to make this determination. This technique has proved useful in identifying separate species in previous work on wide hybridization (Kuhlman et al., 2010).

Senescence of Certain Genotypes

Prior to the harvest of the yield trial, plants in multiple plots had died. These genotypes started to senesce in August 2013 (11 months after planting) and by December 2013, the whole plot was dead. Upon examination, the plots with significant plant death were all the amphidiploids derived from the intergeneric F_1 L07-9s. The effect was observed in both generation one and two and in both replications. In addition,

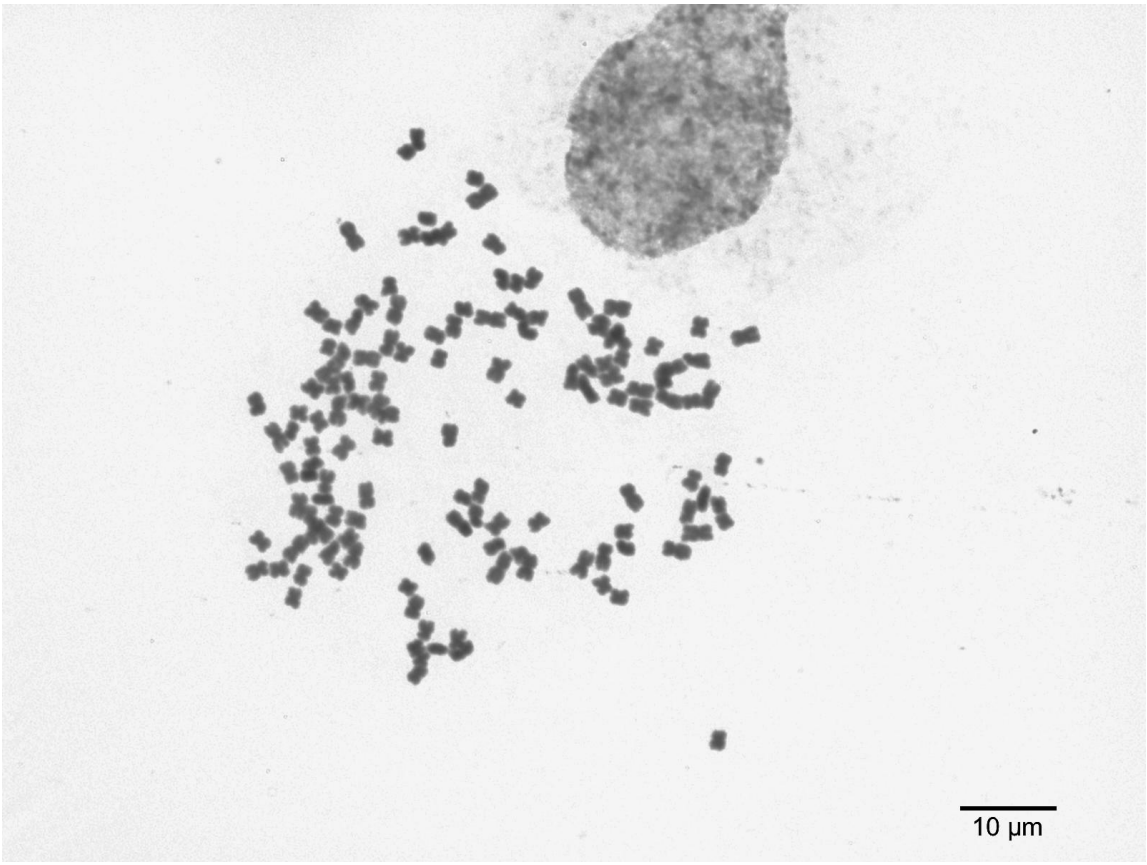

Figure 2. Mitotic chromosome spread of genotype 11BAD23 showing 128 chromosomes at metaphase.

11BAD30 in both replications and 12BAD57 and 12BAD58 in the second replication died before harvest. All other plots were healthy at harvest. The yield trial was treated like commercial sugarcane, with billets planted one fall and the resulting biomass harvested the next fall. The amphidiploid plants remained alive and growing throughout the winter, removing cold temperatures and reduced day length as possible causes of the senescence of the plants.

Sugarcane is a predominately outcrossing species, and self-pollination leads to the expression of deleterious alleles that would normally be masked in a heterozygous state (Ferreira et al., 2005). The intergeneric F_1 genotypes in this experiment contain a haploid set of chromosomes from both sugarcane and sorghum. It is expected that any genotypes carrying strong deleterious alleles would have been automatically screened out in the initial stages of growth following planting of the intergeneric F_1 seed. Only the most vigorous intergeneric F_1 plants were selected for advancement and doubling with colchicine. It seems that the deleterious alleles present in the sugarcane genome are not lethal in the three intergeneric F_1 plants in this study, but cause reduced growth and senescence once the genome is doubled. This phenomenon may occur due to a dosage effect of the deleterious alleles that are causing the premature senescence (Guo et al., 1996). Under this hypothesis, the deleterious allele is present in the intergeneric F_1 but expression levels are not sufficient for lethality. Once the genome is doubled, the deleterious allele is present in two copies and expression levels are doubled or more, leading to quantities sufficient for senescence. All genotypes resulting from L07-9s appear to carry these alleles, however only a few of the amphidiploids from the other

two intergeneric F_1's died prematurely (11BAD30, 12BAD57, and 12BAD58). It is possible that somaclonal variation resulting from tissue culture propagation of the genotypes during treatment with colchicine caused chromosome aberrations that resulted in some genotypes possessing the deleterious alleles while others had lost that segment of DNA. This can occur due to aneuploidy, deletions, translocations, and changes in the epigenetic signaling within the genome (reviewed by Larkin and Scowcroft, 1981). The deleterious alleles present in the genotypes in this study do not manifest themselves until late in the life of the plants. It is likely that the processes causing senescence are triggered by an attempt to enter the reproductive phase. Premature plant death was not observed for any of the genotypes in the greenhouse while material was being increased for the planting of the yield trial. However, the material in the greenhouse was never allowed to grow as long as it did in the field in Weslaco.

Agronomic Results

Significant variation among genotypes was discovered for each agronomic trait evaluated (Table 4). The fresh weight, dry weight, stalk weight, juice weight, and sugar yield of the intergeneric F_1's was significantly higher than the amphidiploid genotypes, indicating that the intergeneric F_1's would likely be more desirable as a bioenergy crop (Table 5). Amphidiploids from the 09BSX4221 family were generally more productive than the other families, indicating that the sugarcane clone used as the male parent has a strong influence on the desirability of the intergeneric progeny (Table 4 and 5). In addition, the 09BSX4221 intergeneric F_1 had significantly greater yields compared to the

Table 4. Mean agronomic performance of each genotype at Weslaco, TX.

Genotype	Pedigree	Height	Lodging	Stand	Uniformity	Brix	Fresh Weight	Dry Weight	Stalk Weight	Juice Weight	Sugar Yield	Moisture
		cm	%	1-5	1-4	%	t ha^{-1}†	t ha^{-1}	t ha^{-1}	t ha^{-1}	kg ha^{-1}	%
11BAD1	L07-9s	77	0	2	1	6.1	4.2	3.2	2.7	1.0	32	23.3
11BAD2	L07-9s	67	0	3	1	11.7	2.2	1.6	1.0	0.6	64	26.7
11BAD3	L07-9s	94	0	1	1	10.3	8.3	5.8	5.8	2.5	210	29.5
11BAD5	L07-9s	100	0	1.5	1	7.6	7.6	5.7	4.1	1.9	129	24.7
11BAD6	L07-9s	100	0	1	1	9.8	8.3	5.4	5.2	2.9	228	35.1
11BAD7	L07-9s	79	0	1	1	8.1	6.8	4.9	3.6	1.9	125	27.4
11BAD8	L07-9s	90	0	1	1	11.2	6.8	5.5	4.5	1.3	114	20.2
11BAD9	L07-9s	88	0	1	1	10.7	10.5	7.9	7.0	2.6	222	24.8
11BAD10	L07-9s	107	0	1.5	1	8.3	10.7	7.6	8.1	3.2	217	29.4
11BAD11	L07-9s	84	0	1	1	8.8	6.1	4.9	4.2	1.2	85	19.5
11BAD13	L07-9s	89	0	1.5	1	11.9	7.3	5.8	5.0	1.6	125	18.3
11BAD15	L07-9s	116	0	1.5	1	11.2	16.4	8.7	11.2	7.6	682	47.0
11BAD16	L07-9s	88	0	1	1.5	8.1	10.7	7.8	7.3	2.9	191	27.0
11BAD22	09BSX4221	187	0	3	1	13.3	9.5	3.2	7.8	6.3	672	65.8
11BAD23	09BSX4221	173	8	2.5	1	11.7	11.7	4.5	8.3	7.2	702	62.0
11BAD30	09BSX5115	77	0	3	1	9.8	2.0	1.3	1.2	0.6	47	30.9
11BAD34	09BSX5115	90	0	3	1	7.8	4.9	2.1	2.3	2.8	120	53.4
11BAD36	09BSX5115	116	0	2	1	10.8	10.7	4.7	5.9	6.1	513	56.1
12BAD41	L07-9s	71	0	2	1	11.8	2.7	1.9	1.3	0.8	114	25.5
12BAD42	L07-9s	64	0	1.5	1	8.0	3.7	2.6	2.3	1.0	65	26.6
12BAD43	L07-9s	91	0	1.5	1	9.3	7.3	5.0	4.8	2.3	168	31.3
12BAD44	L07-9s	93	0	1	1.5	12.4	11.2	6.9	8.7	4.3	450	38.3
12BAD45	L07-9s	94	0	1	1	11.2	9.3	5.8	5.8	3.5	312	36.4
12BAD46	L07-9s	80	0	1.5	1	7.5	4.2	3.6	3.0	0.6	36	13.9
12BAD47	L07-9s	93	0	1	1	8.8	9.8	6.3	7.1	3.5	245	32.9
12BAD48	L07-9s	93	0	1	1	9.2	7.1	4.9	4.2	2.1	159	30.2
12BAD49	L07-9s	91	0	2	1	8.7	8.8	5.8	5.6	3.0	209	33.9
12BAD50	L07-9s	95	0	1	1	7.1	11.5	7.5	6.3	4.0	305	33.9
12BAD51	L07-9s	89	0	2	1	8.8	4.4	3.0	2.1	1.4	74	30.4

Table 4 continued

Genotype	Pedigree	Height	Lodging	Stand	Uniformity	Brix	Fresh Weight	Dry Weight	Stalk Weight	Juice Weight	Sugar Yield	Moisture
		cm	%	1-5	1-4	%	t ha^{-1}	t ha^{-1}	t ha^{-1}	t ha^{-1}	kg ha^{-1}	%
12BAD52	L07-9s	110	0	1	1	8.2	10.5	6.0	8.4	4.5	299	42.9
12BAD53	L07-9s	88	0	2.5	1	9.7	3.9	2.7	2.8	1.2	85	29.7
12BAD54	09BSX4221	180	3	2	1	9.8	16.1	5.7	10.2	10.4	825	64.4
12BAD55	09BSX4221	193	0	2.5	1	11.8	10.0	3.7	7.9	6.4	602	63.3
12BAD57	09BSX5115	83	0	3.5	1.5	10.2	2.0	1.4	1.2	0.6	47	30.5
12BAD58	09BSX5115	76	0	3.5	1	10.2	3.4	1.9	1.7	1.5	109	40.5
12BAD59	09BSX5115	77	0	2.5	1.5	9.0	8.5	3.6	3.9	4.9	376	56.6
L07-9s	F_1	161	0	2	1	13.4	23.9	9.3	18.5	14.7	1579	61.3
09BSX4221	F_1	255	10	1.5	1	15.1	58.1	20.4	51.0	37.8	4566	64.7
09BSX5115	F_1	179	0	3	1	9.4	17.3	6.1	12.8	11.2	849	64.5
LSD (P<0.05)‡		27	5.7	0.9	0.4	4.3	9.2	3.5	7.5	6.0	865	12.8

†Metric tons per hectare

‡Fisher's least significant difference at the 0.05 probability level.

Table 5. Mean agronomic performance by pedigree.

Pedigree	Height	Lodging	Stand	Uniformity	Brix	Fresh Weight	Dry Weight	Stalk Weight	Juice Weight	Sugar Yield	Moisture
	cm	%	1-5	1-4	%	t ha^{-1}†	t ha^{-1}	t ha^{-1}	t ha^{-1}	kg ha^{-1}	%
L07-9s	90b‡	0.0b	1.4c	1.0a	9.4b	7.7bc	5.3b	5.1bc	2.4c	201b	29.2c
09BSX4221	183a	2.5a	2.5ab	1.0a	11.6a	11.8b	4.3bc	8.5b	7.6b	700b	63.9a
09BSX5115	87b	0.0b	2.9a	1.2a	9.6b	5.2c	2.5c	2.7c	2.7c	202b	44.7b
F_1	199a	3.3a	2.2b	1.0a	12.6a	33.1a	11.9a	27.4a	21.2a	2331a	63.5a
LSD (P<0.05)	17	2.3	0.6	0.2	1.8	6.1	2.4	5.3	3.8	510	7.9

†Metric tons per hectare

‡Means within a column followed by the same letter were not significantly different at the 0.05 probability level based on Fisher's least significant difference test.

other intergeneric F_1's for fresh weight, dry weight, stalk weight, juice weight, and sugar yield, indicating that the performance of the intergeneric F_1 may be a good indicator of amphidiploid performance (Table 4). The sugarcane clones used as parents in the original crosses were not available for use as checks. It is possible that the agronomic performance of the sugarcane clone is directly correlated with the agronomic performance of the intergeneric F_1 and amphidiploid. Additional research will be needed to determine if such a correlation exists.

Randomization resulted in the sugarcane plots residing next to substantially shorter material from the amphidiploids. The absence of competition for space and sunlight resulted in greatly inflated biomass yields for the sugarcane checks. The yields obtained were not representative of what would normally be expected for the region and were not suitable for meaningful comparisons to the yields of the sorghum × sugarcane material. As a result, the sugarcane data was not used. However, fresh weight yields for elite sugarcane clones planted adjacent to the yield trial averaged 100 tons per hectare during the 2012-13 growing season (J. da Silva, personal communication, 2014). Although a direct comparison between sugarcane and the sorghum × sugarcane material was not possible, it is apparent that the yields of the amphidiploids and intergeneric F_1's are nowhere close to those typically observed for sugarcane (Table 5).

Lodging was an issue for the intergeneric F_1's and amphidiploids in the 09BSX4221 family, likely due to their increased height compared to the other genotypes. The stand density of several of the amphidiploid genotypes was low, particularly those resulting from 09BSX4221 and 09BSX5115, indicating a lack of vigor and low tillering

ability (Table 4). The majority of the amphidiploids resulting from L07-9s tillered prolifically, which is a desirable trait for increasing yield in sugarcane (Silva et al., 2008). Some minimal variation within plots was observed but this may be due to environmental influences such as soil variability and was not severe enough to attribute to somaclonal variation. The brix values for all sorghum × sugarcane genotypes showed significant variation with readings as high as 15.1 (Table 4). However, the relatively high brix must be coupled with high biomass in order for sugar production to be economically feasible. Continued selection for amphidiploid genotypes with increased brix and fresh weight yield potential will be necessary in order for the sorghum × sugarcane material to be competitive with existing bioenergy species.

Moisture content among the amphidiploids varied considerably due to the senescence of some genotypes (Table 4). Amphidiploids resulting from L07-9s (all of which died before harvest) ranged in moisture content from 13.9 to 47.0%. Although low moisture content would be desirable for ethanol production from the biomass, the premature senescence that led to the reduced moisture is undesirable for total yield and extractable sugar yield.

Composition

The calibration curve used to predict the composition of this material was designed for sorghum and thus, there is likely some error in the estimates of the traits measured. However, the predictions should be consistent across genotypes and allow for comparisons to be made among individuals. Analysis revealed significant variation

among genotypes for each trait (Table 6). Significant variation among amphidiploid genotypes indicates that selection for compositional traits may be effective. The elevated levels of sucrose in the biomass and juice for the intergeneric F_1's and the 09BSX4221 family are indicative of higher sugar content in the stalk. Overall, the mean values for biomass sample composition are similar for the amphidiploids and the intergeneric F_1's, suggesting that doubling the genome does not have the same deleterious effect on plant composition as it does on agronomic traits (Table 5 and 7).

Comparison Between Generations

Contrasts were made between generation one and generation two for each amphidiploid in an effort to determine if potential somaclonal variation from vegetative propagation had any discernible effect on phenotype (Table 8). Very few significant differences were detected between generation one genotypes and their counterparts in generation two. Comparison of the mean performance of all amphidiploids in generation one versus generation two reveals no significant differences for any of the traits analyzed (Table 8).

The agronomic results reinforce the conclusions made based on cytogenetic analysis of several amphidiploid pairs; that somaclonal variation due to vegetative propagation is not occurring at levels high enough to warrant concern. Since only a subset of genotypes were analyzed with chromosome counts, it is possible that some of the other genotypes may be exhibiting considerable variation in chromosome numbers between generations. If so, the variation caused by these chromosome abnormalities is

Table 6. Mean composition of each genotype at Weslaco, TX.

Genotype	Pedigree	Ash	Protein	Sucrose	Lignin	Glucan	Xylan	Galactan	Arabinan	Cellulose	Sucrose	Glucose	Fructose
		----------Biomass----------									---------Juice---------		
		----------%----------											
11BAD1	L07-9s	7.2	2.3	4.4	15.7	35.7	17.9	1.2	3.2	35.5	22.3	18.4	14.7
11BAD2	L07-9s	6.9	3.0	3.4	16.2	34.5	17.9	1.3	3.5	34.1	47.4	6.1	7.0
11BAD3	L07-9s	6.9	2.4	3.4	16.0	35.5	18.2	1.2	3.2	35.3	36.1	13.7	9.2
11BAD5	L07-9s	6.6	2.4	3.3	16.2	35.1	18.2	1.2	3.3	34.9	22.0	13.8	7.0
11BAD6	L07-9s	6.4	2.5	3.1	16.3	34.8	18.3	1.2	3.4	34.4	41.4	11.0	8.1
11BAD7	L07-9s	6.8	2.5	3.6	16.0	35.4	18.1	1.2	3.3	35.1	26.4	18.4	12.4
11BAD8	L07-9s	6.8	2.3	3.3	16.1	35.7	18.2	1.2	3.2	35.3	31.2	8.8	2.3
11BAD9	L07-9s	6.9	2.6	3.3	16.0	35.1	18.1	1.2	3.2	35.0	36.2	14.8	12.7
11BAD10	L07-9s	7.7	2.8	3.8	15.2	34.9	17.8	1.2	3.0	35.0	31.0	16.1	9.3
11BAD11	L07-9s	7.1	2.4	3.5	15.9	35.5	18.2	1.2	3.1	35.8	30.1	12.4	8.1
11BAD13	L07-9s	7.1	2.7	3.8	15.6	35.3	17.8	1.2	3.2	34.9	47.3	11.4	7.0
11BAD15	L07-9s	6.1	2.2	4.2	16.2	34.9	18.2	1.2	3.4	34.2	51.7	15.9	6.6
11BAD16	L07-9s	6.8	2.5	3.7	15.9	34.7	18.1	1.2	3.3	34.6	24.1	14.7	9.1
11BAD22	09BSX4221	5.7	2.8	7.3	13.5	33.3	16.0	1.1	3.3	29.3	56.2	12.0	11.5
11BAD23	09BSX4221	5.9	2.6	6.5	14.0	33.7	16.5	1.2	3.5	30.8	46.1	16.0	11.0
11BAD30	09BSX5115	7.2	2.1	4.3	16.3	36.5	18.3	1.4	3.8	35.6	32.5	17.2	12.8
11BAD34	09BSX5115	7.3	2.0	4.9	15.7	36.0	18.0	1.3	3.8	35.2	22.7	8.2	9.2
11BAD36	09BSX5115	7.7	2.5	4.9	14.7	35.8	17.4	1.3	3.5	34.3	46.0	9.9	6.0
12BAD41	L07-9s	7.2	2.3	4.5	15.3	35.5	17.7	1.2	3.1	35.1	42.1	12.4	6.1
12BAD42	L07-9s	6.7	2.7	3.6	16.0	35.5	17.9	1.2	3.4	34.9	20.6	14.2	10.1
12BAD43	L07-9s	7.0	2.7	3.2	15.7	34.7	18.0	1.2	3.2	34.6	33.9	16.1	11.4
12BAD44	L07-9s	6.1	2.5	3.3	16.2	34.9	18.1	1.2	3.5	33.8	44.9	12.2	7.8
12BAD45	L07-9s	6.1	2.5	3.0	16.4	34.7	18.3	1.2	3.5	34.1	43.8	6.8	5.9
12BAD46	L07-9s	7.1	2.6	3.3	16.1	35.4	18.1	1.2	3.2	34.9	23.1	13.8	9.2
12BAD47	L07-9s	6.6	2.5	3.1	16.1	35.2	18.2	1.2	3.3	34.8	33.6	10.3	7.3
12BAD48	L07-9s	6.7	2.6	3.1	16.1	35.1	18.2	1.2	3.2	34.8	22.9	10.3	8.6
12BAD49	L07-9s	6.5	2.5	3.3	16.3	34.9	18.2	1.2	3.3	34.6	20.8	17.8	9.5
12BAD50	L07-9s	6.6	2.5	3.2	16.3	35.1	18.3	1.2	3.4	35.0	31.3	1.8	6.4
12BAD51	L07-9s	6.5	2.5	3.2	16.2	35.2	18.2	1.2	3.3	34.4	37.5	18.9	8.9

Table 6 continued

Genotype	Pedigree	Ash	Protein	Sucrose	Lignin	Glucan	Xylan	Galactan	Arabinan	Cellulose	Sucrose	Glucose	Fructose
		----------Biomass----------									---------Juice---------		
		----------%----------											
12BAD52	L07-9s	6.7	2.5	3.8	15.8	34.7	18.0	1.2	3.3	34.5	27.7	12.8	9.2
12BAD53	L07-9s	7.1	2.5	3.6	15.4	35.1	17.9	1.2	3.1	34.9	35.4	18.7	14.9
12BAD54	09BSX4221	5.2	2.1	7.4	14.1	33.9	16.6	1.1	3.4	30.9	45.7	3.9	4.1
12BAD55	09BSX4221	6.3	2.8	5.6	13.9	34.2	16.7	1.1	3.2	31.4	39.6	16.4	9.4
12BAD57	09BSX5115	7.3	1.9	4.6	16.4	36.8	18.4	1.3	3.8	36.2	45.5	12.7	9.1
12BAD58	09BSX5115	7.2	2.3	4.6	16.0	36.0	18.0	1.3	3.8	35.1	30.5	8.3	7.8
12BAD59	09BSX5115	7.1	2.3	4.6	15.5	36.0	17.8	1.3	3.7	34.7	25.2	11.3	7.4
L07-9s	F_1	5.6	2.8	5.6	14.3	33.5	16.9	1.2	3.4	31.1	57.5	8.8	6.4
09BSX4221	F_1	5.8	3.0	6.8	12.9	33.5	15.8	1.0	3.0	30.2	73.4	17.0	11.7
09BSX5115	F_1	6.3	2.2	4.9	14.7	35.5	17.6	1.2	3.5	33.6	34.5	16.9	11.8
LSD (P<0.05)†		0.8	0.6	1.1	0.8	1.1	0.6	0.1	0.3	1.8	27.7	11.1	8.3

†Fisher's least significant difference at the 0.05 probability level.

Table 7. Mean composition by pedigree.

Pedigree	Ash	Protein	Sucrose	Lignin	Glucan	Xylan	Galactan	Arabinan	Cellulose	Sucrose	Glucose	Fructose
	----------Biomass----------									----------Juice----------		
	----------%									----------		
L07-9s	6.8b†	2.5a	3.5d	16.0a	35.1b	18.1a	1.2b	3.3b	34.8a	33.5b	13.3a	9.0a
09BSX4221	5.8c	2.6a	6.7a	13.9b	33.8c	16.5c	1.1c	3.4b	30.6c	46.9a	12.1a	9.0a
09BSX5115	7.3a	2.2b	4.7c	15.8a	36.2a	18.0a	1.3a	3.7a	35.2a	33.7b	11.3a	8.7a
F_1	5.9c	2.7a	5.8b	14.0b	34.2c	16.8b	1.1c	3.3b	31.6b	55.1a	14.2a	10.0a
LSD (P<0.05)	0.4	0.3	0.5	0.5	0.5	0.3	0.1	0.2	0.8	11.4	4.6	3.2

†Means within a column followed by the same letter were not significantly different at the 0.05 probability level based on Fisher's least significant difference test.

Table 8. Contrasts between generation 1 and 2 for all traits.

Generation 1	Generation 2	Height	Lodging	Stand	Uniformity	Brix	Fresh Weight	Dry Weight	Stalk Weight	Juice Weight	Sugar Yield	Moisture	Ash	Protein	Sucrose†	Lignin	Glucan	Xylan	Galactan	Arabinan	Cellulose	Sucrose‡	Glucose	Fructose
11BAD1	12BAD41																							
11BAD2	12BAD42			*																				
11BAD3	12BAD43																							
11BAD5	12BAD44				*	*						*												
11BAD6	12BAD45																							
11BAD7	12BAD46											*												
11BAD8	12BAD47																							
11BAD9	12BAD48																							
11BAD10	12BAD49												*			*								
11BAD11	12BAD50											*												
11BAD13	12BAD51																							
11BAD15	12BAD52																							
11BAD16	12BAD53			*	*			*																
11BAD22	12BAD54			*										*				*						
11BAD23	12BAD55		*																					
11BAD30	12BAD57				*																			
11BAD34	12BAD58											*												
11BAD36	12BAD59	*			*																			
Mean Generation 1	Mean Generation 2																							

*Significant at the 0.05 probability level.
†Sucrose composition of the biomass.
‡Sucrose composition of the juice.

not severe enough to translate into a large phenotypic difference for the traits measured in this study.

Conclusion

Based on this study, neither the intergeneric F_1's nor the amphidiploid intergeneric hybrids have yield potentials required of a biomass crop. The three intergeneric F_1 genotypes demonstrated superior performance for most traits compared to the amphidiploids. This does not mean that amphidiploids could not be produced that perform as well or better than their intergeneric F_1 parents. The sample size studied in this experiment is relatively small for logistical reasons, and analysis of a larger number of intergeneric F_1's and their amphidiploids may produce genotypes with a mean performance similar to or better than the intergeneric F_1. The material in this study resulted from the first hybrids obtained between sorghum and sugarcane. With additional crossing and selection for agronomic traits, it may be possible to increase the desirability of sorghum × sugarcane hybrids and amphidiploids.

Since the intergeneric F_1 hybrids between sorghum and sugarcane are male and female sterile, creating amphidiploids is essential to obtain fertile plants for backcrossing to sorghum or sugarcane. Backcrossing would be beneficial if the sorghum and sugarcane genomes undergo recombination and the progeny could be selected for greater agronomic desirability. Alternatively, backcrossing to one parent or the other would maintain the diploid chromosome number of the recurrent parent while resulting in a

haploid number of chromosomes from the non-recurrent parent. Additional research in this area may produce interesting results.

The variation in mean values among amphidiploid families for the majority of the traits indicates that the sugarcane clone has a large effect on the productivity of the amphidiploid genotypes resulting from the cross since the same sorghum inbred was used for all intergeneric F_1 crosses (Table 5 and 7). In light of this, the use of a diverse set of sugarcane accessions in additional sorghum × sugarcane crosses may reveal patterns in combining ability such that more targeted crosses may be attempted. The sorghum inbred used as the female was bred for grain production characteristics, traits which are not necessarily desirable for a sorghum × sugarcane hybrid (Kuhlman and Rooney, 2011). The use of an elite sweet sorghum inbred homozygous for *iap* may be beneficial in order to obtain sorghum × sugarcane hybrids with enhanced agronomic potential.

The sugarcane clones used as male parents for the three F_1 hybrids in this study were not available for use as checks. Analysis of the performance of the sugarcane parent alongside the intergeneric F_1 may reveal a correlation between performance of the two, such that the most elite sugarcane clones may produce more desirable sorghum × sugarcane hybrids. Alternatively, exotic accessions that are largely unimproved by breeding or selection may prove to be more valuable. Additional research of this kind will need to be performed in order to begin identifying such associations.

CHAPTER V

FINE MAPPING OF THE *Iap* LOCUS

Introduction

Hybridization between cultivated sorghum [*Sorghum bicolor* (L.) Moench] and divergent sorghum species has been hampered until recently by pollen-pistil incompatibilities (Hodnett et al., 2005; Price et al., 2005). The mutant sorghum gene *Inhibition of Alien Pollen (iap)* overcomes the fertilization barriers imposed by the wild type form of the gene, allowing pollen tubes of several closely related species and genera to reach the ovary of sorghum that is homozygous for the *iap* allele (Bartek et al., 2012; Hodnett et al., 2010; Kuhlman et al., 2008; Laurie and Bennett, 1989; Price et al., 2006). The mutant *iap* gene was discovered by Laurie and Bennett (1989) when screening sorghum accessions for the ability to allow maize pollen tube growth in the stigmas. One accession from China, Nr481, allowed maize pollen tube growth into the ovary although no hybrid plants were recovered. The *iap* gene was bred into sorghum germplasm adapted to the southern U.S. and is present in homozygous form in Tx3361 (Kuhlman and Rooney, 2011). In intergeneric crosses with sugarcane (*Saccharum* spp.), Hodnett et al. (2010) successfully produced 14,141 seeds in crosses between Tx3361 and several commercial and exotic sugarcane clones. After performing embryo rescue as necessary, 1,371 hybrid plants were grown and screened for relevant agronomic traits. Morphology of these hybrid plants is similar to sugarcane although the panicles are more

compact than is observed in sugarcane (Hodnett et al., 2010). The hybrid plants are male and female sterile and methods are being attempted to mitigate the sterility (G. Hodnett, personal communication, 2012). Previous attempts to hybridize sorghum and sugarcane have been met with little success (De Wet et al., 1976; Bourne 1935; Nair 1999). The frequency of hybrids has been much higher in attempts utilizing sorghum homozygous for the *iap* allele and has led to improvements in the techniques used to produce hybrid plants. Premature harvesting of the seed, excision of the pericarp, and embryo rescue have led to seedling recovery rates as high as 33% (Hodnett et al., 2010). This new technology has the potential to allow for rapid and efficient introgression of novel traits from both sorghum and sugarcane into a new hybrid crop.

Cross-incompatibility genes have previously been identified in other crop species. In wheat (*Triticum aestivum* L.), cross-incompatibility with related species is controlled by genes kr_1-kr_4 and by *SKr* (Alfares et al., 2009). In maize (*Zea mays* L.) several crossability genes have been identified as well. *Teosinte crossing barrier1* (*Tcb1*) prohibits cross-pollination by individuals carrying the *tcb1* gene. The system works reciprocally i.e. male pollen that contains *Tcb1* is rejected on females that carry *tcb1* and females that contain *Tcb1* reject pollen that is carrying *tcb1* (Evans and Kermicle, 2001). In the *Ga1* system in maize, the interactions are different. According to Evans and Kermicle (2001), if the female is homozygous *Ga1-s* and is pollinated with homozygous *ga1* pollen, no seed will be produced. When a female homozygous for *Ga1-s* is pollinated with a mixture of *Ga1-s* and *ga1* pollen, the *ga1* pollen is rejected. However, when a female homozygous for *ga1* is pollinated with either *Ga1-s* or *ga1* pollen, full

seed set results in both cases. To date, no cross-incompatibility genes have been cloned in wheat or maize, although a fine mapping study has been performed for the *SKr* gene in wheat (Alfares et al., 2009).

Several proteins in tomato have been discovered that regulate the process of pollen-pistil compatibility. LeSTIG1 is expressed by the stigma and binds to LePRK2, a receptor kinase present on the pollen coat (Tang et al., 2004). LeSTIG1 was shown to promote pollen tube growth after germination. The presence of LeSTIG1 was not required for pollen tube growth, but pollen tubes were much longer and had a greater chance of reaching the ovary with LeSTIG1 (Tang et al., 2004). Tomato has a wet stigma and LeSTIG1 is present in the stigma exudate that directly interacts with the pollen coat. On a species such as sorghum with dry stigmas, the pollen grain must be compatible for hydration and germination to occur (Swanson et al. 2004). It is likely that different processes govern pollen-pistil interactions in species with a dry stigma as little overlap in signaling cues has been found between the two systems (Hiscock and Allen, 2008). In species with dry stigmas, most of the research on pollen-pistil interactions has focused on self-incompatibility and unilateral incompatibility systems, and very little is known about the systems governing interspecific incompatibility (Hiscock and Allen, 2008; Bernacchi and Tanksley, 1997).

In order to realize the full potential of the *iap* gene it must be bred into different sorghum genetic backgrounds depending on the desired end result of the wide cross. For example, an elite sweet sorghum line with high sugar content could potentially result in a sorghum × sugarcane hybrid with a sugar content similar to the original sugarcane parent.

Currently, the phenotyping required to track the gene is labor and time intensive and is subject to possible environmental influences (Kuhlman and Rooney, 2011). In order to more efficiently track the gene it would be desirable to have a molecular marker completely linked to the gene for breeding. The *iap* locus has been mapped to the short arm of chromosome 2 by Kuhlman (2007), but a marker closer to the locus is needed. Another avenue of investigation would be to fine map and clone the *iap* gene to determine if it shares any homology with genes in other crop species. Identification of a previously unidentified homolog in another species could lead to additional intergeneric crosses and introgression of traits between other crops.

The purpose of this study is to fine map the *iap* locus and identify a candidate gene(s) based on phenotype and molecular markers.

Materials and Methods

Plant Material

The population used for mapping the *iap* locus is a BC_1F_1 population created by crossing Tx3361*Ms3ms3* (Tx3361) (Kuhlman and Rooney, 2011) as the female to BTx623*Ms3ms3* (BTx623), followed by backcrossing the F_1 to Tx3361. Seed of Tx3361 and BTx623 was planted in Weslaco, TX in the fall of 2010. Both parents were segregating for male fertility due to the *Ms3* gene. Male-sterile panicles of Tx3361 were identified and bagged at the onset of anthesis to prevent foreign pollen contamination. A single male-fertile panicle of BTx623 was used to pollinate a single male-sterile panicle of Tx3361. Several of these crosses were made and at maturity the Tx3361 panicle that

set the most seed was selected for advancement. F_1 seed from this single panicle was planted in a row in College Station, TX in the spring of 2011. Male-sterile plants of Tx3361 were identified and bagged to prevent cross-pollination. Male-fertile F_1 plants were used as the male parent in the backcross to the bagged male-sterile Tx3361 plants. At maturity, seed was harvested from the female plants and bulked to form the BC_1F_1 mapping population.

Genotyping

Individual BC_1F_1 plants in the mapping population were identified with a unique number and were tissue sampled after approximately two weeks of growth. Each tissue sample was placed in a 1.2ml tube containing a stainless steel rod. 450 µl of buffer working solution was pre-heated to 65°C and added to each tube.

Buffer working solution:

-45% lysis buffer stock: 0.2M Tris pH 7.5, 0.05M ethylenediaminetetraacetic acid (EDTA), 2M NaCl, 2% CTAB

-10% Sarcosyl stock: 5% w/v sodium lauroyl sarcosine

-45% extraction buffer stock: 0.026M sodium metabisulfite, 0.5mM polyvinylpyrrolidone (PVP), 0.35M Sorbitol, 0.1M Tris pH 7.5, 5mM EDTA

Tissue samples were ground using a Spex CertiPrep Genogrinder 2000 (Metuchen, New Jersey) at 1400 strokes per minute for 100 seconds followed by a 30 minute incubation at 65°C. 450 µl of phenol:chloroform:isoamyl alcohol (25:24:1) was added to each tube followed by vigorous agitation for five minutes. Samples were spun down in a Beckman Coulter Allegra 6KR centrifuge (Brea, CA) for 30 minutes at 1924 x g. Approximately 400 µl of supernatant was transferred to a new tube where 450 µl chloroform:isoamyl alcohol (24:1) was added. Samples were inverted 40 times and spun down for 30 minutes at 1924 x g in the centrifuge. Following centrifugation, 400 µl of supernatant from each sample was transferred to a 96 well deep well plate. 450 µl of pure isopropanol at a temperature of -20°C was added to each well followed by pipetting up and down 10 times to mix. The plates were centrifuged for 90 minutes at 1924 x g, the supernatant was discarded, and the remaining pellet was washed with 400 µl of 70% ethanol. The plates were centrifuged for 15 minutes at 1924 x g and the remaining supernatant was discarded. The pelleted DNA was allowed to air dry at room temperature before being resuspended in 100 µl millipure sterile water. One µl of RNase Cocktail Enzyme Mix (Ambion, Grand Island, NY) was added to each well in order to degrade any residual RNA. DNA concentration was quantified using the AccuBlue Broad Range dsDNA Quantitation Assay (Biotium, Hayward, CA) and read on a Victor X3 2030 Multilabel Reader (Perkin Elmer, Waltham, MA). Based on the quantification results, each DNA sample was diluted to approximately 1-10 ng/µl. Samples were stored at -20°C until they were needed.

The PCR reaction for each sample was: 2 µl 5X buffer, 1 µl MgCl, 0.8 µl dNTP's, 1 µl forward and reverse primers labeled with either HEX or FAM fluorescent dyes, 0.04 µl Taq polymerase, 3.16 µl millipure sterile water, and 2 µl diluted DNA. DNA plates were run on a GeneAmp PCR System 9700 thermocycler (Applied Biosystems, Carlsbad, CA) under the following conditions: 95°C for two minutes followed by 30 cycles of 95°C for 45 seconds, 55°C for 45 seconds, and 72°C for 45 seconds. The program finished with 20 minutes at 72°C and a 4°C hold until the plates were removed. One µl of PCR product from each sample was combined with 5 µl Genescan 400HD (ROX) size standard/Hi-Di Formamide (Applied Biosystems, Carlsbad, CA) in a 96 well plate. The plates were analyzed on an ABI 3130 Genetic Analyzer (Applied Biosystems, Carlsbad, CA) followed by analysis using GeneMapper version 4.0 (Applied Biosystems, Carlsbad, CA).

Phenotyping

The phenotyping process used for this research project required pollinating the sorghum stigmas with maize pollen as described by Laurie and Bennett (1989). The variety 'Kandy Korn' was used as the pollen source since it was shown by Bartek et al. (2012) to have the highest incidence of pollen tube growth into the sorghum ovary out of the maize accessions tested. At the onset of anthesis, the sorghum panicles were checked for male sterility. The *Ms3* marker is linked to the *Ms3* locus, although based on our observations in our mapping population the marker is approximately 15.8 cM from the locus, resulting in several fertile plants due to crossover events between the

marker and locus. In the fall of 2011, the sterile panicles were not bagged to prevent foreign pollen since the 'Kandy Korn' was the only source of pollen in the greenhouse, airflow was minimal, and the fertile panicles were bagged and removed once fertility was confirmed. In the spring of 2012, sterile panicles were bagged at the onset of anthesis due to many sources of sorghum pollen in the greenhouse and a greater amount of airflow.

Once the male-sterile sorghum panicles had completely flowered, fresh maize pollen was collected and gently brushed onto the stigmas using a small paintbrush. At least 100 stigmas were pollinated from each plant and a large load of pollen was applied. On each day that one or more plants from the mapping population were pollinated, a single male sterile Tx3361 and ATx623 plant were also pollinated as controls. Twenty-four hours after pollination, pollinated florets were harvested and placed into glass vials containing 3:1 (95% ethanol : glacial acetic acid) fixative for a minimum of one week. The pistils were extracted from the florets and placed in 70% ethanol at -20°C until needed. The pistils were processed according to the protocol described by Kho and Baer (1968) with modifications as described by Hodnett et al. (2005). Slides were analyzed with a Zeiss Universal II microscope (Carl Zeiss Inc., Gottingen, Germany) with a 10X Neofluor objective. Fluorescence of the callose in the pollen tubes was induced using 390-420 nm light emitted from a mercury lamp with a 450 nm emission filter.

Results and Discussion

Fall 2011

To keep the number of plants manageable for phenotyping and genotyping, 384 BC_1F_1 seeds were planted in each replication in the greenhouse. Two replications were planted one week apart in the fall of 2011 with the first planted on September 23, 2011. The seeds were planted in standard greenhouse flats consisting of 36 cells per flat with one seed planted in each cell. Five pots each of Tx3361 and ATx623 were planted on the same day each replication of the mapping population was planted. Tx3361 (*iapiap*) was used as the positive control and ATx623 (*IapIap*) was used as the negative control. The ensure the maize and sorghum plants flowered at roughly the same time for phenotyping, 10 pots of maize variety 'Kandy Korn' were planted each week in the greenhouse beginning two weeks before the mapping population was to be planted and continuing for two to three weeks after the last replication of the mapping population had been planted. Standard watering and fertilization practices were observed.

Samples were screened for the *Ms3* genetic male sterility gene using the simple sequence repeat (SSR) marker Sb03QGM269 provided by David Jordan (personal communication) (Table 9). The mapping population was segregating 1:1 for male fertility:sterility due to the male fertile F_1 with genotype *Ms3ms3* being used to pollinate male sterile Tx3361 plants homozygous recessive for *ms3*. Samples were analyzed on an ABI 3130 Genetic Analyzer (Life Technologies, Grand Island, NY) followed by analysis using GeneMapper v4.0 (Life Technologies, Grand Island, NY). A total of 703 BC_1F_1 plants were sampled and genotyped for the *Ms3* male sterility gene. Three

Table 9. Forward and reverse primer sequences (5'-3') for SSR and INDEL markers used in mapping the *iap* locus in Fall 2011 and Spring 2012.

Marker Name	Forward Primer	Reverse Primer
Sb03QGM269	GCATGGCCAGCCTCTCGATGG	TCGCTGCAGAACGCAGGTGG
CS051	ACGGACGGGAACAGAGAAAGAA	ACGAGGACGAGTGCATGATGAG
Txp80	GCTGCACTGTCCTCCCACAA	CAGCAGGCGATATGGATGAGC
Txp50	TGATGTTGTTACCCTTCTGG	AGCCTATGTATGTGTTCGTCC
Txi84	GTCAGGGGAAGAAGGGAAGA	GCCATTGCCTGCTAGCTACT
Txi87	GTGTTGCCTTGTTGAGGTGA	TACCCTGTTTGCAGCTTGTG
Txi88	TCCCAATGCCTCCTCAATAA	TGAGGGACATGGCTTCCTAC
Txi89	GGATGGAATGGAACCCTAGC	CACCACATTCCACAGCACAG
Txi90	TACTGATGACCACGCCAATG	CGCAGGATCTGTTGTTGTTG
Txi91	ATCCAGGTGCGTCAAGTCTG	ACCCAAAAATTCCTCCTTGC
Txi92	GGCTGCAAAAATAGAT	GCAGCCTCCTTTTAATTTGC

hundred and twenty-five individuals were identified that were homozygous recessive for the *Ms3* allele and were re-arrayed in new 96-well plates.

Sequences determined to be polymorphic between the two parents were used to create markers in the putative *iap* locus region identified by Kuhlman (2007). Three SSR's (CS051, txp80, and txp50) and one INDEL (txi84) spanning roughly three million base pairs (bp) were polymorphic between the two parents and were used to screen the selected sterile individuals (Table 9). The four markers in the *iap* region identified 76 plants with a crossover out of the 325 male sterile individuals identified. From each replication six plants with the genotype *iapiap* were selected as a positive control and six plants with the genotype *IapIap* were selected as a negative control for phenotyping to ensure the genomic region under investigation contained the *iap* locus. Selected individuals were transplanted from the greenhouse flats to three-gallon pots for phenotyping. Two plants were identified as being male fertile after the plants began flowering and were excluded from further analysis.

The standards for determining whether or not a plant was homozygous for the *iap* allele were taken from Kuhlman (2007). Twenty-four intact pistils were observed from each individual plant. A plant was classified as being homozygous for *iap* if a maize pollen tube was observed in the ovary in at least one of the pistils and heterozygous at the *iap* locus if no maize pollen tubes were found in the style of any of the 24 pistils. Due to possible genotypic and environmental variation, a pollen tube was not observed in the ovary on all 24 of the pistils for any of the BC_1F_1 plants or for Tx3361, the positive control homozygous for *iap*. The negative control, ATx623, showed

germination of some of the pollen although no pollen tubes reached the stigma axis. The BC_1F_1 plants selected as controls with the genotype *iapiap* or *IapIap* had maize pollen tube phenotypes corresponding to their genotypes, confirming that the region in question contained the *iap* locus. The phenotypes of the plants and the corresponding location of crossovers between the four markers were used to narrow the region around the *iap* locus. Based on this data, it was determined that the *iap* locus was between 1990816 bp (CS051) and 2799632 bp (txi84) on the short arm of chromosome 2. Sixteen plants contained a crossover between these two markers and were used for further analysis.

Spring 2012

Based on the phenotyping results from the fall of 2011, the genomic region was narrowed from three million bp to approximately 800,000 bp. DNA was extracted from a bulk of several plants of Tx3361 and BTx623 using a Fast DNA Spin Kit (MP Biomedicals, Santa Ana, CA). The parental DNAs were used to make Digital Genotyping (DG) template libraries (Morishige et al., 2013) and the DG templates were subjected to genotyping by sequencing on an Illumina Hi-Seq 2000 (Illumina Inc., San Diego, CA). The sequence reads from the two parents were independently mapped to the BTx623 reference genome. Reads from each parent that mapped to the identical location within the *iap* locus on the reference genome were then compared to identify additional INDELs in the region. Eleven INDELs were identified between CS051 and txi84 with six (txi87-92) showing adequate amplification and polymorphism between the two parents (Table 9).

Additional BC_1F_1 seeds were planted weekly beginning January 5, 2012. Controls and the maize were planted as described previously. A total of 2,897 plants were sampled and genotyped for the *Ms3* marker as described previously. Flanking markers CS051 and txi84 were used to screen the 1,467 sterile individuals and 80 individuals with a crossover were identified. Due to fertility resulting from crossovers between the *Ms3* locus and the *Ms3* marker, 13 plants were fertile and could not be phenotyped. An additional four plants either did not flower or had questionable phenotype results and were excluded from further analysis.

Pollen tube growth on the positive control (Tx3361) did not meet the standards previously used. It was observed that pollen adhesion on the stigma branches was very poor and pollen tubes rarely grew into the style or ovary. This lack of pollen tube growth was similar to but more severe than that observed by Kuhlman (2007). Genotypic variation was ruled out since the same seed source for Tx3361 was used for both the fall 2011 and spring 2012 plantings. The difference in humidity between the fall and spring was hypothesized to be the cause for the difference in phenotypes observed. The fall of 2011 was very dry following a record drought in central Texas while the winter and spring of 2012 were fairly wet and humid. It was observed that the air felt very humid in the greenhouse when pollinations were being made and when florets were harvested the following day. This high humidity and temperature was not observed in the fall of 2011.

A different standard for phenotyping was developed to overcome the variation in pollen tube growth. A plant was determined to have the genotype *iapiap* if at least one

pollen tube grew into the stigma axis on two or more pistils. A plant was designated as heterozygous at the *iap* locus if no pollen tubes on any of the 24 pistils grew into the stigma axis. None of the pistils on the negative control (ATx623) showed pollen tube growth into the stigma axis, assuring that there were no false positives. The BC_1F_1 plants selected as controls with the genotype *iapiap* or *IapIap* had pollen tube phenotypes corresponding to their genotypes, confirming that the region in question still contained the *iap* locus. Based on the phenotyping of the 80 individuals with a crossover, the region containing *iap* was reduced to approximately 124,000 bp.

DNA from 11 individuals and the two parent lines was extracted using Fast DNA Spin Kits (MP Biomedicals, Santa Ana, CA) and DG template libraries were prepared and run on the Illumina Hi-Seq 2000. The sequence reads from all 11 progeny and the two parent lines were individually mapped to the BTx623 reference genome. Following mapping, the data was examined across the *iap* locus. Nine of the 11 individuals had a crossover between CS051 and txi84, one individual had the parental genotype of Tx3361 between CS051 and txi84, and the other individual was heterozygous at all markers between CS051 and txi84. The sequencing data further refined the crossover breakpoints, narrowing the putative region to approximately 80,000 bp. This region contains eight genes according to the *S. bicolor* reference genome version Sbi1.4 (Paterson et al., 2009).

Spring 2013

The DG data produced from the parental lines was further screened for the presence of SNPs within the refined *iap* locus. Nine SNPs were identified in the region and the sequence information was used to design markers for the KBiosciences Competitive Allele-Specific PCR genotyping system (KASP). Sequences containing a polymorphic SNP were BLASTed against the S. *bicolor* reference genome using Phytozome v8.0 (Goodstein et al., 2012) to obtain 200-400 bp flanking the 72 bp sequence read from the Illumina. Flanking and allele specific primers were designed using Batch Primer 3 (You et al., 2008) under the following specifications: minimum primer size of 12 mer, optimum and maximum of 30 mer; minimum primer melting temperature of 52°C, optimum of 57°C, and maximum of 63°C; minimum product size of 0 mer, optimum of 100 mer, and maximum of 1000 mer. Primer sequences were analyzed for possible hairpin structures, self-annealing, and 3' complementarity using OligoCalc (Kibbe 2007). The selected primer sets were BLASTed against the S. *bicolor* reference genome using Phytozome v8.0 (Goodstein et al., 2012) in order to screen out primers with a high probability of non-specific binding. The BLASTN program was used on a non-masked genome with an expect (E) threshold of 10; default settings were used for all other parameters. Primer sets exhibiting the fewest number of negative characteristics were selected with one marker being located between each gene in the refined *iap* locus (Table 10). The KASP assay was performed as described by Robinson et al. (2011) with Tx3361 as the positive control and BTx623 as the negative control. A total of 5,515 BC_1F_1 plants were sampled and genotyped for the flanking SNP markers

Table 10. Flanking and allele specific primer sequences (5'-3') for the KASP assay in Spring 2013.

Marker	Flanking Primer	Allele Specific Primer (*Iap*)	Allele Specific Primer (*iap*)
A	AAAACGAGAAGAGAAGAAAAGAAG	ATAGATATAGTTTCTAGTTTTGGGACTGT	ATATAGTTTCTAGTTTTGGGACTGC
B	ATTAAGTTCAAACTGTCCACTAAATAATAG	TAAATCTCTCGTTCTTTGTTCTTTTAC	TAAATCTCTCGTTCTTTGTTCTTTTAT
C	GTGTGTGCAATGTCAACAATATC	CATCCACGAAATGTTCACG	AACATCCACGAAATGTTCACT
D	GTCTACTAAACGAAAGCCAAGTTTAT	TAAAAAAATTCCAAGAAGGGC	CTAAAAAAATTCCAAGAAGGGA
E	CTAATTTGTATAGTCTTGAGGAGATAGAAT	GTGTTTGGTTCTTTGGAATTT	TGTGTTTGGTTCTTTGGAATTA
F	TATCCAACTAGACAATCTACTATCACATAC	ATAGAAATGTGACTTGCTGACA	TATAGAAATGTGACTTGCTGACG
G	TTATAGAAGATGGGAATTTAGATACAAC	AACATACTACCAAATAACCACATAACA	CATACTACCAAATAACCACATAACG
H	CAACAAACATACTCCACTGTAGC	CTCCTGCTACAGAATGAGGTTA	TCCTGCTACAGAATGAGGTTG
J	TGTAATAAAATAACAAAAGGAAGAAAACT	TTTTACTTTAGGGCTTTTATTTTTTC	TTTACTTTAGGGCTTTTATTTTTTT

using the KASP assay (Figure 3). Due to the narrow genomic region being analyzed, the *Ms3* marker was not used in order to keep as many individuals with a crossover as possible. Seven individuals were identified with a crossover between markers A and J (Table 10). Four of the plants were fertile and were hand-emasculated two days before pollinating with maize pollen. The individuals were phenotyped as previously described. Based on the phenotyping and genotyping data, the region was narrowed to approximately 48,000 bp. Three genes are present in this region according to the *S. bicolor* reference genome version Sbi1.4: Sb02g002170.1 (2170), Sb02g002180.1 (2180), and Sb02g002190.1 (2190) (Paterson et al., 2009). Gene 2170 is described as a predicted protein, 2180 is described as similar to a putative uncharacterized protein, and 2190 is described as weakly similar to a putative uncharacterized protein (Paterson et al., 2009). None of the three genes are obvious candidates based on their proposed function.

Next Generation Sequencing

Tx3361 was re-sequenced following construction of Illumina Tru-Seq DNA libraries using a 100bp paired-end strategy at the University of Texas Genomic Sequencing and Analysis Facility (P. Klein, personal communication). The paired reads were uploaded to CLC Workbench v6.0 and mapped to the reference genome as well as subjected to a *de novo* assembly. Upon aligning the Tx3361 sequence reads to the sorghum reference genome sequence (BTx623), it was observed that multiple gaps were present in the Tx3361 sequence compared to BTx623 in the 48,000 bp region of interest. It was hypothesized that these gaps may have resulted either from insertions or deletions

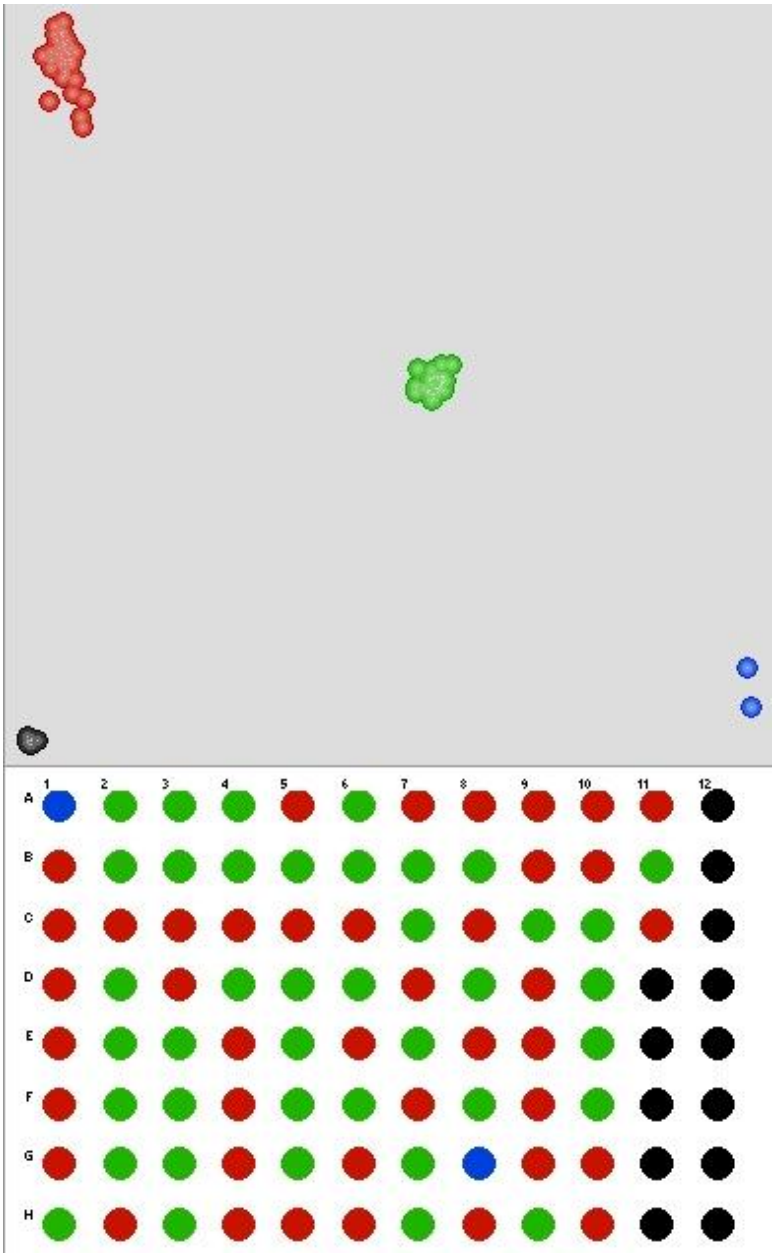

Figure 3. Output from the KASP assay showing the genotype of each BC_1F_1 individual in a 96-well plate. Cell A1 is BTx623 and cell B1 is Tx3361 (controls). Red samples were homozygous for the Tx3361 version of the SNP, green samples were heterozygous, blue samples were homozygous for the BTx623 version of the SNP, and black samples were blank controls.

in Tx3361 relative to the reference sequence. To test this hypothesis, primers were designed flanking each of the gaps in the region of interest (Table 11). These seven sets of primers were used to amplify Tx3361 and Tx623 genomic DNA. The parental DNAs were diluted to 5 ng/µl and used for PCR amplification. The following PCR program designed for large amplicons was used: 94°C for 1 minute followed by 35 cycles of 94°C for 50 seconds, 55°C for 50 seconds, and 72°C for 4 minutes. The program ended with 72°C for 20 minutes followed by a 4°C hold. PCR products were run on a 0.8% agarose gel stained with ethidium bromide for 1.5 hours. Analysis of the results revealed that some of the gaps in Tx3361 are actually present while others are not. A combination of insertions and deletions of Tx3361 sequence relative to the reference sequence are responsible for the gaps observed. One deletion in particular is of interest; the deletion is 624 bp total and removes 106 bp from the end of gene 2180 (Iap3, Table 11). This gene is present in the *S. bicolor* reference genome version Sbi1.4 (Paterson et al., 2009), but has been removed in the latest reference version 2.1 at Phytozome.net for unknown reasons. One of the insertions is noteworthy due to the size of the apparent insertion (Iap5, Table 11). The gap in Tx3361 is approximately 3,300 bp compared to the reference genome sequence. Analysis of the bands on the gel reveals a possible 1,800 bp insertion in addition to the 3,300 bp gap already discussed. This 5,100 bp region is large enough to conceal a gene in Tx3361. However, since the lack of a pre-zygotic barrier is expressed when the *iap* allele is homozygous recessive, it is likely that the phenotype results from the loss of function of an existing gene present in cultivated sorghum. Under this hypothesis, a gene present in the reference genome has been

Table 11. Flanking primer sequences (5'-3') for gaps in the Tx3361 sequence compared to the reference genome sequence.

Marker	Forward Primer	Reverse Primer	Amplicon Size (bp)	Gap Size (bp)
Iap1	GCCATGTAAGCATAGCATAGAG	CAGACCACATACATATTGACCA	655	270
Iap2	GACACTGTTGGCTGATTCAA	CATCTGGTGTACCAAACCAC	475	169
Iap3	ATCGGCCAGATAGACAAGAG	ACTCCACCGGCTTACTAACA	885	624
Iap4	ATCAACTTTGCCTCCACCT	TGCAGGTTAGATTTTAGGGTTT	477	200
Iap5	TAGAAAGGACAAGCCGACTC	GATTAAGCAGCACGTCACAC	3926	3300
Iap6	CCTTGGTTTTGAATGTCCTC	CAACTCGAACGTCCCTTCT	1351	459
Iap7	GAAACTTTTGACACCGATGC	TTTCTTTTGCTCGTGATCG	858	652

mutated, resulting in the *iap* allele. Therefore, the candidate gene should be present in both Tx3361 and the reference genome (BTx623).

Characterization of Gene Function

The genomic sequence of each gene as well as 2 kb upstream and downstream from the gene were extracted from the CLC Genomics Workbench. The sequence for BTx623 was obtained from the reference genome sequence and the sequence for Tx3361 was from the re-sequencing data. All subsequent analyses were performed using prediction programs available from Softberry (http://www.softberry.com).

Sb02g002170.1

The genomic sequence of gene 2170 as well as 2 kb upstream and downstream were extracted for BTx623 and Tx3361. Alignment of the sequences using SCAN2 (Softberry) revealed high sequence homology overall. The sequences were annotated using FGENESH (Softberry) with default parameters. The software identified two putative genes within the sequence for BTx623 and three genes for Tx3361 (Table 12 and 13). Two of the predicted proteins were similar between BTx623 and Tx3361, but contained insertions in the protein sequence for Tx3361 that are significant enough to cause a change in secondary structure according to output generated by PSSFinder (Softberry). The third predicted gene in Tx3361 did not have a homolog in BTx623 and comparisons could not be made.

Table 12. Sequences (5'-3') for predicted proteins in the region of Sb02g002170.1 in BTx623.

Exons	Chain	Amino Acids	Sequence
4	Forward	197	MTSSQPPPGQPLSMPHLEHGMGARVSTAGGGAASRSNVTEAVVSEGRRFQ EPIHRVAWSPLSSDSYMPMSKKANSSQKRSTFGSEPEEAYASTSRAGAASY AAGMPYREMSRSAPEPERSLASYFKAALLFFLLVDKVDDGGVGGVRACDW LARAPMVAEKRNSYESATTSTQSREVSNVNSALWWPEPPDLSPKQV
2	Reverse	623	MSTAVPLVAGSNYNVTDTDEGRLAARCDPEFQSPVVIVWASLVQIIMINTS AIVAVDDREGRNIGPPFDLLVQGLWTYYLGVTYFTNNSGINSRDGLITFVVL IIGLEGIPFALTCAKMVFKYYAFEKARQSFALGNNPHLIFAYMRQPPPVQAR TSHRREAAAVTNEDAPPPPPLVVMGEGKRNMEKQSHGYVWKGDSGMAVH GKDGLVTIDSVWKMTACFFPFSTLQRLQDLCLSFAFFKLLRCRFAGYNVHT ANASSDMFTFFWSLLLKEGDHNRVRDMASYVCSNWTKVALVCRLLNRAS SSSSKHSLCIQKCAGLLIRCRCKLLRRHWDEKIGQFSVLVLQPRATPLGLLW YLFPFLPDQNRKVSVPAVVKVCIMQALRRTRDDGGQLSNGAACLRRCRDL GESFNWACSNKSTSYTILTWHIATSILEVRYPNLNDEGQGSPPLSNTDYKMV ATHLSRYCAYLVTWCPELLPDDHEWSRSLYENVKKDTRRVLAGCTAGDSL TQEVKCQQLVELLSTKAKHQVVKDGARLGEQLAKLVLHGGDDTAWKLLA EFWSEMVLYVAPSDNLKGHKEAIARGGELITLLWVLLFHAGIVSRPGENNS AAAAAAAATSAAGVV

Table 13. Sequences (5'-3') for predicted proteins in the region of Sb02g002170.1 in Tx3361.

Exons	Chain	Amino Acids	Sequence
4	Forward	210	MTSSHPPPGQPLSTPHLEHSMGARVSTAGGGAASRSNVTEAVVSEGRRFQEPIRRIAWSP CVPRGGGTSAQNRLSSDSYMPVSKKANSSPKRSTFGSESEEAYASTSRAGAASYAVGTP YREMSRSVLEPELSLASNFKAALLFFLLVDKVDDGGVGGVRGCDWLARAPMAAEKRK SYESATTSTQSREVSNVNSALWWPEPPALSPKQV
1	Reverse	818	MAGGNSTRDHCSYNALKQCSSRIICDGQSLSLFHKSIRHRLWLVNALLVASAILAGVIV GIGIFGQRYRYHRLTRFIFVGATTLFLPVMCTAVPLVAGSNYYVTDTDEGRLAARCDPE FQSPVVIVWASLVQIIMINTSAIVAVDDREGRNIGPPFDLLVQGLWTYYLGVTYFTNNSG INSRDGLITFVVLIIGLEGIPFALTCAKMVFKYYAFEKARQSFALGNNPHLIFAYMRQPPP VQARTSHRREAAAVTNEDAPPPPPLVVMGEGKRNMEKQSHGYVWKGDSGMAVHGKD GLVTIDSVWKMTACFFPFSTLQRLQDLCLSFAFFKLLRCRFAGYNVNTANASSDMFTFF WSLLLKEGDHNRVFRLISEEISFVHDYYYTSIPISYSKCWLPIVGIFISLLSITYCILAATWL SFLLAVNTGSPQLLCFISCTENQLQTQLDIRLYGSWLFGLVPLLFLLVLVFITEVRDMTSY VCSNWTKVALVCRFLNRASSSLSSSKHSLCIQKCAGLLIRCRCKLLRRHWDEKIGQFSV LALQPRATPFGILWYLFPFLPDQNRKVSVPAVVKVCIMQALRRTRDDGGQLSNGAACL RRCRDLGESFNWACSNKSTSYTILTWHIATSILEVRYPHLNDEGQCSPPLSNTDYKMVA THLSRYCAYLVTWCPELLPDDHEWSRSLYENVKKDTRRVLAGCTAGDSLTQEAKCQQ LVELLSTKAKHQVVKDGARLGEQLAKLVLHGDDDTVWKLLAEFWSEMVLYVAPSDN LKGHKEAIARGGELITLLWVLLFHAGIVSRPGENNSAAAAAAAATSAAGVV
3	Reverse	79	MPALATGMLTLVVVHLPIVVGASLRSQRRQRRLYGSNGEAAFLRLIPAFDEDGHNRPLP LPKHEDGDGASQPVGVKILA

BLASTP with default parameters was used to search for homology in protein sequences within the grass family. One of the homologous sequences between BTx623 and Tx3361 returned hits for "hypothetical protein" in the database, but the results did not provide additional information on gene function. The other protein sequences did not contain similarity to any known proteins in the grass family.

Sb02g002180.1

The genomic sequence of gene 2180 as well as 2 kb upstream and downstream were extracted for BTx623 and Tx3361. The sequence for Tx3361 contains a 624 bp deletion compared to BTx623, 106 bp of which are deleted from the end of the second exon of 2180. Alignment of the sequences using SCAN2 revealed good homology overall with the exception of the large deletion in Tx3361. Annotation of the sequences from each genotype using FGENESH with default parameters revealed three predicted genes in BTx623 and one predicted gene in Tx3361 (Table 14 and 15). The predicted gene in Tx3361 is composed of eight exons spanning approximately 3 kb while the three predicted genes in BTx623 are composed of one, two and three exons, respectively.

Alignment of the three predicted proteins from BTx623 with the protein from Tx3361 using SCAN2a (Softberry) revealed very little homology. It is likely the deletion in Tx3361 has altered the sequence enough to cause a total change or loss of function of 2180. BLASTP with default parameters did not reveal significant homology between any of the predicted protein sequences and known grass family proteins.

Table 14. Sequences (5'-3') for predicted proteins in the region of Sb02g002180.1 in BTx623.

Exons	Chain	Amino Acids	Sequence
1	Forward	73	MEHYRRETRRAAQDAARGHTMDGREFFKRRLAGHLHRAGTLFAASSARASATSHATE APAGDNLPLLAHLLGR
2	Forward	133	MAGHDPTTAGPDQAIVGLDLATVGLDSSTTDEPRARCGSRRPRSTASTSNVPLRYDDPM FLESSLSVGPVVGPCYFSNSSGDDPVSPGSGVKPQSLGKSHEGSTKTSDEAHMEQDLYE GRALSDGGLPADDDG
3	Forward	364	MVVFVSPSARDVRCLRAILECFTGALGLVTNTDKCQASPIRCSAEEMALVRRAFPCRITP FPCKYLGVPLSIYRLHRAEEQTLVDIVAMKIPTWKSGLLMSTGRVLLTKVTLSAIQVHV VIASCLSQWAIGQIDKRRRAFLWTGKDSVSRGKCKLAWKTVCLPTANRGLGMIDLRLF GYALRLRWEYCVLSNLTEDGIGSLCSFVPLLYNGISRAECGRSIQDALNNRQWVRDIVG PTTIQVMCQYIKTWLLVWGTALDPLRSDCFIWKWSLDGNYSVSSTYRAFFVGSTKLLG AKELLHTRAPLKMWKERNTRTFRNENSTQMAVAKRIHDEGAQWIDEGFSTLASFWTSV GDVGLVAQSMAV

Table 15. Sequence (5'-3') for the predicted protein in the region of Sb02g002180.1 in Tx3361.

Exons	Chain	Amino Acids	Sequence
8	Forward	476	MALGHGIGPRGAVSTGVVAQDLQLAGPSLAMAGYDPTTAGLDQAIVGLDLATVGLDS ATTMSLELDAAAYDDPMFLESSLSAGPVVDPCYFSNSSGDDPVSPGLIKELLAPRATPPP HTQAKDLLAPVLLNPQTVPQLVTGRCIHDNFRTVSLTCKWLIKVDMAKAFDSVAWPFL LDLLRHIGFPQRWTDLLSITLSSASPKPLINGQPGCRILHAHGLRQGDPMSPMLFVIVME VLNSMIVEADRQQILFRLHRAEEQPLVDTVAVKIPTWKSGLLTSAGRALLTKVTLSAIQ VHVVITSCLSQWAIGQIDKRRRAFLWTGKDSVSRGKCKLAWKMVTVSGATAGVGAFD SLVLLVSWDMWKERNARTFTNESSTLMVVAQCIHDEGAQWIDEGFSTLASFWTSVGD VGLVAQSMAVYCPKSNQTHEEADRGREKEDECIYGNGARSPVMRCDPPSPEKMASQQ SSEIYWLGSSS

Sb02g002190.1

The genomic sequence of gene 2190 as well as 2 kb upstream and downstream were extracted for BTx623 and Tx3361. Analysis of the alignment of the BTx623 and Tx3361 sequences using SCAN2 revealed mostly strong homology, although long stretches of identical sequence were frequently interrupted by small insertions and deletions in Tx3361. Annotation of the sequences from each inbred using FGENESH with default parameters revealed two predicted genes in both BTx623 and Tx3361 (Table 16 and 17). The first predicted protein in BTx623 is read from the forward strand while the first predicted protein in Tx3361 is read from the reverse strand. Although the two predicted genes are in a similar location in the genome, there is no homology between their protein sequences. The second predicted protein in BTx623 and Tx3361 share considerable homology, although the beginning and end of the transcripts do not correspond and there is a deletion of 34 amino acids in the middle of the Tx3361 protein relative to the homologous protein in BTx623. Analysis of the secondary structure of the two homologous predicted proteins using PSSFinder revealed structural changes that would likely result in a difference in tertiary structure, and therefore function, of the two predicted proteins.

BLASTP with default parameters against known proteins in the grass family did not find any homology for the first predicted protein in BTx623 and Tx3361. The second predicted protein in both BTx623 and Tx3361 shared homology with known grass family proteins, however they were “hypothetical proteins” and did not provide additional information on putative gene function.

Table 16. Sequences (5'-3') for predicted proteins in the region of Sb02g002190.1 in BTx623.

Exons	Chain	Amino Acids	Sequence
2	Forward	134	MKRLSTSPLSPSMGLLSPIHRCCCPTTGVPPLPPSTSLSSLPVAEKGTLRARCPLRRRGGE EWIRVVLAMDPRCPRRIRPLMPEDGGTVVDIPGAGKGGKGARTSTKGYTCRRGGEEAR VVEIPGAGRSVVVQD
3	Reverse	725	MRLRLWLVNALLLAGAILGGVIVGIGVFGQRYRYHRLTRIIFLGATALFLPVMSTVVPL VAGSNDYVTVGQSGMRSQLAAKCDPGVQSTLVVIWASLVQIIMINTSAIVAVDDREGR NIGPPFQLLVQGIWTFYLGTSYVISDAIGGGDLKFVRFTTGIEGIPFALTCAKIFFKYYAFE KARRSFALGNNPHLIFAYMQQPPPVQAGTSHRREAAVVTDDDAPPPPPLLVMGEGKRH MEKQSHGYVWKGDSGHGKDGLVTIDSVWKMTAFLPFSTLQRIKDTCLSFAFFKLLRCR FARYNINTANASSDMFTFFWSLLLKDGGQNRVFQVISDEISFVHDYYYTSIPISYSKCWL PIVVFVAEVKDMASFICSNWTKVALVCHLLNRASSSSSSSSKNSLCIQKCAGLLLRCRCK LLRTHWDEKIGQCSVLVLQPRASLLGILWHLFPLLPDKKRKVRVPAAVKLCIIQALRRT RDDGGQLSNGAACLRRRRGQVGESFNWACSNRSTSYTILTWHIATSILEVRYPHLNDEG QGSPPLSNTAYKMVATHLSRYCAYLVTWCPELLPDDHEWSRSLYENVKKDTRRVLAG CTAGDSLTSEAKCQQLVELLSTEAKHEVVKDGAKLGEQLGELVLEGGDDTAWKLLAE FWSEMILYVAPSDNLKGHKEAIARGGELITLLWVLLFHAGIVSRPGEDDGAAAATSAAD SAVLVDVFLQLSAFELSLF

Table 17. Sequences (5'-3') for predicted proteins in the region of Sb02g002190.1 in Tx3361.

Exons	Chain	Amino Acids	Sequence
2	Reverse	127	MQPPRPHGRPLPSPTLPLHSQPPCAPPSLPDSFPPLAAPMRALFPPSHASPLILDDDAPPHA WDLHDARLLPPSPTCVSLRRRACPLPSLSRAWDIHDGAPILRHERPDPARTARIHGEDNA DLPLS
2	Reverse	696	MAGGNSTGDHCSYDALSLFDKSMRLRLWLVNALLLAGAILGGVIVGIGVFGQRYRYHR LTRIIFLGATTLFLPVMSTVVPLVAGSNDYVTVGQSGMRSQLAAKCDPGVQSTLVVIWA SLVQIIMINTSAIVAVDDREGRNIGPPFQLLVQGIWTFYLGTSYVINDAIGGGDLKFVRFT TGIEGIPFALTCAKIFFKYYAFEKARRSFALGNNPHLIFAYMQQPPPVQAGTSHRREAAV VTDEDAPPPPPLLVMGEGKRHMEKQSQGYVWKGDSGHGKDGLVVTIDSVWKMTAFLP FSTLQRIKDTCLSFAFFKLLRCRFARYNINTANASSDMFTFFWSLLLKDGGQNRVFQVKD MASFICSNWTKVALVCHLLNRASSSSSSSKNSLCIQKCAGLLLRCRCKLLRTHWDEKIG QCSVLVLQPRASLLGILWHLFPLLPDKKRKVRVPAAVKVCLIQALRRTRDDGGQLSNGA ACLRRRRGQVGESFNWACSNRSTSYTILTWHISTSILEVRYPHLNDEGQGSPPLSNTDYK MVATHLSRYCAYLVTWCPELLPDDHEWSRSLYENVKKDTWRVLAGCTAGDSLTSEAK CQQLVELLSTEAKHEVVKDGAKLGEQLGELVLEGGDDTAWKLLAEFWSEMILYVAPSD NLKGHKEAIARGGELITLLWVLLFHAGIVSRPGEDDGAAAATSAAGVV

Conclusion

It is not surprising that meaningful sequence homology was not discovered between the three candidate genes and known proteins in the grass family. In order to protect the identity of a species after speciation, a great amount of diversity in incompatibility mechanisms is necessary (Allen et al., 2010). Extensive research on rice (*Oryza sativa*) has elucidated the function of many genes, however sorghum and rice diverged approximately 50-70 million years ago (Roulin et al., 2009). It is likely that a sufficient amount of time has passed for the sorghum incompatibility mechanism to evolve beyond recognition compared to similar mechanisms in rice and other grasses. Due to the lack of homology between incompatibility genes among species, it is likely that other methods will need to be employed in order to clone the *Iap* gene. Sequencing of the transcriptome has been an effective method for identifying genes that regulate specific processes in sorghum (Dugas et al., 2011). Sequencing of the RNA transcripts produced by pollinating BTx623 and Tx3361 with maize pollen should identify differential expression of the candidate genes and result in the identification of the *Iap* gene. Additionally, sequencing of the entire transcriptome may identify other genes or modifiers that play a role in alien pollen rejection. Identification of mutant forms of these other genes may result in the success of pollinations with sorghum and additional species.

Once the *Iap* gene is identified, future work should focus on identifying the mutation that renders the wild type form of the gene non-functional. This may provide insight on the function of the wild type form of the gene. Knowledge of the function of

the gene could be used to find similar genes in other species since insufficient sequence homology exists to use the sequence of the gene alone to find homologs.

CHAPTER VI

CONCLUSIONS

Utilization of the mutant *iap* allele has facilitated the rapid development and screening of thousands of sorghum × sugarcane hybrids. The male and female sterility of these intergeneric F_1 hybrids necessitated the production of amphidiploids using colchicine in order to have fertile plants for continued crossing and selection. A replicated yield trial of multiple amphidiploid genotypes and intergeneric F_1 hybrids revealed that the amphidiploids possess inferior agronomic potential compared to the intergeneric F_1's. Analysis indicates that neither the intergeneric F_1's nor the amphidiploid intergeneric hybrids have yield potentials required of a biomass crop. Genetic variation among the amphidiploid families for many of the traits indicates that the sugarcane parent has a large effect on the phenotype of the intergeneric hybrid. Continued crossing and selection could produce progeny with more desirable agronomic performance. In addition, the use of a sweet sorghum inbred homozygous for *iap* as the sorghum parent may be beneficial in order to obtain intergeneric hybrids with agronomic potential approaching that required of a bioenergy crop. Although early yield results do not appear promising, perseverance should be emphasized.

Maximum maize pollen tube growth on the pistils of Tx3361 was achieved at 45% humidity, the lowest humidity tested. The results demonstrate that lower humidity is desirable for obtaining maize pollen adhesion and germination on sorghum stigmas

homozygous for *iap*. Since significant amounts of pollen tube growth to the sorghum ovary were not observed, it is possible that even lower humidity levels are necessary. Maize pollen was the subject of this research, however the results suggest that the sensitivity to environment is caused by differences in expressivity of the *iap* allele. As a result, future intergeneric crosses utilizing sorghum homozygous for *iap* should take place in an environment where the humidity is naturally low during an extended period of time.

The region containing the *Iap* gene has been reduced to approximately 48 kb on the short arm of chromosome 2. Three putative genes reside in this segment, although none have been annotated based on homology with known grass family proteins. Analysis of predicted proteins in the region around each candidate gene revealed very little homology in predicted protein sequence between Tx3361 and the reference genome sequence. In addition, meaningful sequence homology was not identified between the three candidate genes and known proteins in the grass family. In order to protect the identity of a species, incompatibility mechanisms evolve at a rapid pace. It appears that sufficient time has passed for the prezygotic barrier represented by *Iap* to evolve beyond recognition compared to homologous mechanisms in rice and other grasses. Sequencing of the transcriptome in response to alien pollen may be necessary in order to identify which of the three candidate genes is *Iap*.

The results presented here provide a strong foundation for facilitating the efficient use of the *iap* allele in future wide crosses with sorghum. Future work should focus on identifying the *Iap* gene and identifying the mutation that renders the wild-type

form of the gene non-functional. Knowledge of the function of the gene could potentially be used to find similar genes in related species since insufficient sequence homology exists to use the sequence of the gene alone to find homologs. Once the function of *iap* has been elucidated, it should be determined how environmental conditions affect the expressivity of the *iap* allele. Additional research may identify other abiotic variables such as temperature that modify the function of *iap*.

REFERENCES

Alfares, W., A. Bouguennec, F. Balfourier, G. Gay, H. Berges, S. Vautrin, P. Sourdille, M. Bernard, and C. Feuillet. 2009. Fine mapping and marker development for the crossability gene *SKr* on chromosome 5BS of hexaploid wheat (*Triticum aestivum* L.) Genetics 183(2):469-481.

Allen, A.M., C. Lexer, and S.J. Hiscock. 2010. Comparative analysis of pistil transcriptomes reveals conserved and novel genes expressed in dry, wet, and semidry stigmas. Plant Physiol. 154:1347-1360.

Al-Janabi, S.M., M. McClelland, C. Petersen, and B.W.S. Sobral. 1994. Phylogenetic analysis of organellar DNA sequences in the Andropogoneae: Saccharinae. Theor. Appl. Genet. 88:933-944.

Baker, J.T., L.H. Allen, and K.J. Boote. 1992. Response of rice to carbon dioxide and temperature. Agric. For. Meterol. 60:153-166.

Ballesteros, J., C. Garcia-Llamas, M.C. Ramirez, and A. Martin. 2003. Low relative humidity increases haploid production in durum wheat x maize crosses. Plant Breeding 122:276-278.

Bartek, M.S. 2013. A study in *Sorghum bicolor*: QTL analysis of photoperiod sensitive sorghums, evaluation of sorghum × sugarcane hybrids and trait introgression for intergeneric hybrid improvement. Ph.D. diss., Texas A&M Univ., College Station.

Bartek, M.S., G.L. Hodnett, B.L. Burson, D.M. Stelly, and W.L. Rooney. 2012. Pollen tube growth after intergeneric pollinations of *iap*-homozygous sorghum. Crop Sci. 52:1553-1560.

Bennett, A.S. and R.P. Anex. 2009. Production, transportation and milling costs of sweet sorghum as a feedstock for centralized bioethanol production in the upper Midwest. Bioresour. Technol. 100:1595-1607.

Berding, N. and B.T. Roach. 1987. Germplasm collection, maintenance, and use. p. 143-210. *In* D.J. Heinz (ed.) Sugarcane improvement through breeding. Elsevier, Amsterdam.

Bernacchi, D. and S.D. Tanksley. 1997. An interspecific backcross of *Lycopersicon esculentum* × *L. hirsutum*: linkage analysis and a QTL study of sexual compatibility factors and floral traits. Genetics 147:861-877.

Bourne, B.A. 1935. A comparative study of certain morphological characters of sugarcane x sorgo hybrids. J. Agric. Res. 50:539-552.

Brar, D.S., and G.S. Khush. 1997. Alien introgression in rice. Plant Mol. Biol. 35:35-47.

Burner, D.M. and M.P. Grisham. 1995. Induction and stability of phenotypic variation in sugarcane as affected by propagation procedure. Crop Sci. 35:875-880.

Campbell, A.W., W.B. Griffin, D.J. Burritt, and A.J. Conner. 2001. The importance of light intensity for pollen tube growth and embryo survival in wheat x maize crosses. Ann. Bot. 87:517-522.

Corn, R.J. 2009. Heterosis and composition of sweet sorghum. Ph.D. diss., Texas A&M Univ., College Station.

De Vicente, M.C. and S.D. Tanksley. 1991. Genome-wide reduction in recombination of backcross progeny derived from male versus female gametes in an nterspecific cross of tomato. Theor. Appl. Genet. 83:173-178.

De Wet, J.M.J., S.C. Gupta, J.R. Harlan, and C.O. Grassl. 1976. Cytogenetics of introgression from *Saccharum* into *Sorghum*. Crop Sci. 16:568-572.

Dugas, D.V., M.K. Monaco, A. Olson, R.R. Klein, S. Kumari, D. Ware, and P.E. Klein. 2011. Functional annotation of the transcriptome of *Sorghum bicolor* in response to osmotic stress and abscisic acid. BMC Genomics 12:514-534.

Engelke, J. 2005. Sugarcane: Measuring commercial quality. Farmnote 23/2002. Dep. of Agriculture, Kununurra, WA, Australia.

Evans, M.M.S. and J.L. Kermicle. 2001. *Teosinte crossing barrier1*, a locus governing hybridization of teosinte with maize. Theor. Appl. Genet. 103:259-265.

Ferreira, F.M., M.H.P. Barbosa, R.D. de Castro, L.A. Peternelli, and C.D. Cruz. 2005. Effects of inbreeding on the selection of sugar cane clones. Crop Breed. App. Biotech. 5:174-182.

Food and Agriculture Organization of the United Nations. 2012. FAOSTAT data. http://faostat.fao.org (Accessed August 4, 2012).

Food and Agriculture Organization of the United Nations. 2013. FAOSTAT data. http://faostat.fao.org (Accessed January 2, 2013).

Foster, C.A. 1968. Ryegrass hybridisation: The effect of artificial isolation materials on seed yield and floral environment. Euphytica 17:102-109.

Goldemberg, J. 2007. Ethanol for a sustainable future. Science 315:808-810.

Goodstein, D.M., S. Shu, R. Howson, R. Neupane, R.D. Hayes, J. Fazo, T. Mitros, W. Dirks, U. Hellsten, N. Putnam, and D.S. Rokhsar. 2012. Phytozome: a comparative platform for green plant genomics. Nucl. Acids Res. 40(D1):D1178-D1186.

Guo, M., D. Davis, and J.A. Birchler. 1996. Dosage effects on gene expression in a maize ploidy series. Genetics 142:1349-1355.

Heslop-Harrison, J. 1975. Incompatibility and the pollen-stigma interaction. Ann. Rev. Plant Physiol. 26:403-425.

Hiscock, S.J. and A.M. Allen. 2008. Diverse cell signaling pathways regulate pollen-stigma interactions: the search for consensus. New Phytol. 179:286-317.

Hodnett, G.L., B.L. Burson, W.L. Rooney, S.L. Dillon, and H.J. Price. 2005. Pollen-pistil interactions result in reproductive isolation between *Sorghum bicolor* and divergent *Sorghum* species. Crop Sci. 45:1403-1409.

Hodnett, G.L., A.L. Hale, D.J. Packer, D.M. Stelly, J. da Silva, and W.L. Rooney. 2010. Elimination of a reproductive barrier facilitates intergeneric hybridization of *Sorghum bicolor* and *Saccharum*. Crop Sci. 50:1188-1195.

Imrie, B.C. 1966. Stigma receptivity in cytoplasmic male sterile wheat. Aust. J. Exp. Agric. Anim. Husb. 6:175-178.

Jaster, J.R. 1985. Analysis of seed set and stigma receptivity among cytoplasmic male sterile sorghum [*Sorghum bicolor* (L.) Moench] cultivars. Master's thesis. Texas A&M Univ., College Station.

Kho, Y.O. and J. Baer. 1968. Observing pollen tubes by means of fluorescence. Euphytica 17:298-302.

Kibbe, Warren A. 2007. OligoCalc: an online oligonucleotide properties calculator. Nucl. Acids Res. 35(suppl 2):W43-W46.

Kuhlman, L.C. 2007. Sorghum introgression breeding utilizing *S. macrospermum*. Ph.D. diss., Texas A&M Univ., College Station.

Kuhlman, L.C., B.L. Burson, P.E. Klein, R.E. Klein, D.M. Stelly, H.J. Price, and W.L. Rooney. 2008. Genetic recombination in *Sorghum bicolor* x *S. macrospermum* interspecific hybrids. Genome 51:749-756.

Kuhlman, L.C., B.L. Burson, D.M. Stelly, P.E. Klein, R.R. Klein, H.J. Price, and W.L. Rooney. 2010. Early-generation germplasm introgression from *Sorghum macrospermum* into sorghum (*S. bicolor*). Genome 53:419-429.

Kuhlman, L.C. and W.L. Rooney. 2011. Registration of Tx3361 sorghum germplasm. J. Plant Reg. 5:133-134.

Kumar, A., R.K. Chowdhury, and O.S. Dahiya. 1995. Pollen viability and stigma receptivity in relation to meteorological parameters in pearl millet. Seed Sci. Technol. 23:147-156.

Larkin, P.J. and W.R. Scowcroft. 1981. Somaclonal variation- a novel source of variability from cell cultures for plant improvement. Theor. Appl. Genet. 60:197-214.

Laurie, D.A. and M.D. Bennett. 1989. Genetic variation in sorghum for the inhibition of maize pollen tube growth. Ann. Bot. 64:675-681.

Lingle, S.E. and T.L. Tew. 2008. A comparison of growth and sucrose metabolism in sugarcane germplasm from Louisiana and Hawaii. Crop Sci. 48:1155-1163.

Moran, J.L. 2000. Differences in ergot vulnerability among sorghum genotypes and the relationship between stigma receptivity and ergot vulnerability. Master's thesis. Texas A&M Univ., College Station.

Morishige, D.T., P.E. Klein, J.L. Hilley, S.M.E. Sahraeian, A. Sharma, and J.E. Mullet. 2013. Digital genotyping of sorghum – a diverse plant species with a large repeat-rich genome. BMC Genomics 14:448-466.

Mujeeb-Kazi, A. 1981. *Triticum timopheevii* x *Secale creale* crossability. J. Hered 72:227-228.

Nair, N.V. 1999. Production and cyto-morphological analysis of intergeneric hybrids of *Sorghum* x *Saccharum*. Euphytica 108:187-191.

Paterson, A.H., J.E. Bowers, and B.A. Chapman. 2004. Ancient polyploidization predating divergence of the cereals, and its consequences for comparative genomics. Proc. Natl. Acad. Sci. USA 101:9903-9908.

Paterson, A.H., J.E. Bowers, R. Bruggmann, I. Dubchak, J. Grimwood, H. Gundlach, G. Haberer, U. Hellsten, T. Mitros, A. Poliakov, J. Schmutz, M. Spannagl, H. Tang,

X. Wang, T. Wicker, A.K. Bharti, J. Chapman, F.A. Feltus, U. Gowik, I.V. Grigoriev, E. Lyons, C.A. Maher, M. Martis, A. Narechania, R.P. Otillar, B.W. Penning, A.A. Salamov, Y. Wang, L. Zhang, N.C. Carpita, M. Freeling, A.R. Gingle, C.T. Hash, B. Keller, P. Klein, S. Kresovich, M.C. McCann, R. Ming, D.G. Peterson, M. Rahman, D. Ware, P. Westhoff, K.F.X. Mayer, J. Messing, and D.S. Rokhsar. 2009. The *Sorghum bicolor* genome and the diversification of grasses. Nature 457:551-556.

Pickering, R.A. 1980. Attempts to overcome partial incompatibility between *Hordeum vulgare* and *H. bulbosum* L. Euphytica 29:369-377.

Prasad, P.V.V., K.J. Boote, and L.H. Allen Jr. 2006. Adverse high temperature effects on pollen viability, seed-set, seed yield and harvest index of grain-sorghum [*Sorghum bicolor* (L.) Moench] are more severe at elevated carbon dioxide due to higher tissue temperatures. Agric. For. Meteorol. 139:237-251.

Prasad, P.V.V., S.R. Pisipati, R.N. Mutava, and M.R. Tuinstra. 2008. Sensitivity of grain sorghum to high temperature stress during reproductive development. Crop Sci. 48:1911-1917.

Price, H.J., G.L. Hodnett, B.L. Burson, S.L. Dillon, and W.L. Rooney. 2005. A *Sorghum bicolor* x *S. macrospermum* hybrid recovered by embryo rescue and culture. Aust. J. Bot. 53:579-582.

Price, H.J., G.L. Hodnett, B.L. Burson, S.L. Dillon, D.M. Stelly, and W.L. Rooney. 2006. Genotype dependent interspecific hybridization of *Sorghum bicolor*. Crop Sci. 46:2617-2622.

Robinson, P. and J. Holme. 2011. KASP version 4.0 SNP genotyping manual. University of Bristol. Accessed November 6, 2012 from http://www.cerealsdb.uk.net.

Rooney, W.L., J. Blumenthal, B. Bean, and J.E. Mullet. 2007. Designing sorghum as a dedicated bioenergy feedstock. Biof., Bioprod., Bioref. 1:147-157.

Rosenow, D.T. and J.A. Dahlberg. 2000. Collection, conversion, and utilization of sorghum. In: C.W. Smith and R.A. Frederiksen, editors, Sorghum: Origin, history, technology, and production. John Wiley and Sons, New York. p. 309-328.

Ross, W.M. 1957. Stigma receptivity in cytoplasmic male-sterile sorghum. Agron. J. 49:219-220.

Roulin, A., B. Piegu, P.M. Fortune, F. Sabot, A. D'Hont, D. Manicacci, and O. Panaud. 2009. Whole genome surveys of rice, maize, and sorghum reveal multiple horizontal transfers of the LTR-retrotransposon *Route66* in *Poaceae*. BMC Evol. Bio. 9:58-67.

SAS Institute. 2011. The SAS system for windows. Release 9.3. SAS Inst., Cary, NC.

Shapouri, H. and M. Salassi. 2006. The economic feasibility of ethanol production from sugar in the United States. USDA, Office of the Chief Economist. http://www.usda.gov/oce/reports/index.htm (Accessed January 30, 2013).

Sharma, H.C. 1995. How wide can a wide cross be? Euphytica 82:43-64.

Silva, M.A., J.A.G. da Silva, J. Enciso, V. Sharma, and J. Jifon. 2008. Yield components as indicators of drought tolerance of sugarcane. Sci. Agric. 65:620-627.

Sitch, L.A. and J.W. Snape. 1987. Factors affecting haploid production in wheat using the *Hordeum bulbosum* system. 1. Genotypic and environmental effects on pollen grain germination, pollen tube growth and the frequency of fertilization. Euphytica 36:483-496.

Spurbeck, J.L., A.R. Zinsmeister, K.J. Meyer, and S.M. Jalal. 1996. Dynamics of chromosome spreading. Am. J. Med. Genet. 61:387-393.

Swanson, R., A.F. Edlund, and D. Preuss. 2004. Species specificity in pollen-pistil interactions. Annu. Rev. Genet. 38:793-818.

Tang, W., D. Kelley, I. Ezcurra, R. Cotter, and S. McCormick. 2004. LeSTIG1, an extracellular binding partner for the pollen receptor kinases LePRK1 and LePRK2, promotes pollen tube growth *in vitro*. Plant J. 39:343-353.

Tukey, J.W. 1949. Comparing individual means in the analysis of variance. Biometrics 5:99-114.

United States Congress. 2007. Energy Independence and Security Act of 2007. http://www.epa.gov/regulations/laws/eisa.html. (Accessed January 2, 2013).

Vanderlip, R.L. and H.E. Reeves. 1972. Growth stages of sorghum [*Sorghum bicolor*, L. Moench]. Agron. J. 64:13-16.

Wolfrum, E., C. Payne, T. Stefaniak, W.L. Rooney, N. Dighe, B. Bean, and J. Dahlberg. 2013. Multivariate calibration models for sorghum composition using near-infrared spectroscopy. Technical Report NREL/TP-5100-56838 pp 1-14.

You, F.M., N. Huo, Y.Q. Gu, M. Luo, Y. Ma, D. Hane, G.R. Lazo, J. Dvorak, and O.D. Anderson. 2008. BatchPrimer3: a high throughput web application for PCR and sequencing primer design. BMC Bioinformatics 9:253.